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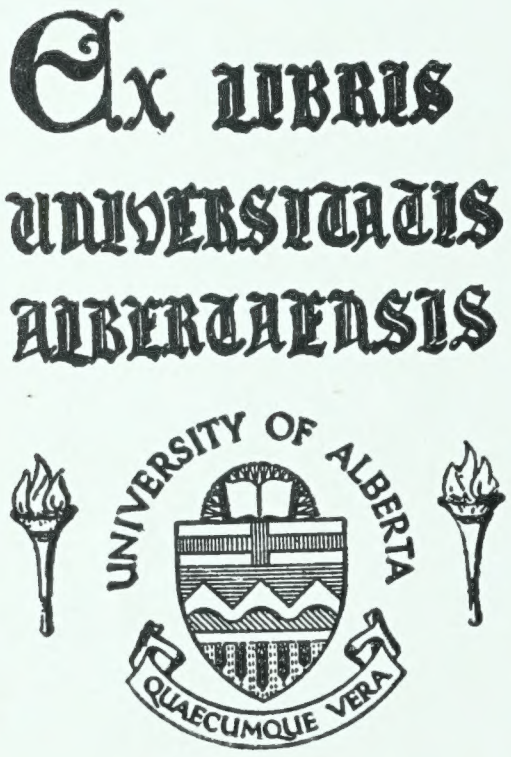

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SOME EFFECTS OF N^6-BENZYLADENINE ON THE MORPHOLOGY AND POST-HARVEST PHYSIOLOGY OF RADISH (*Raphanus sativus* L.) AND CABBAGE (*Brassica oleracea* L. var. *capitata* L.)

FACULTY OF AGRICULTURE

DEPARTMENT OF PLANT SCIENCE

by

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EDMONTON, ALBERTA

APRIL, 1964.

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Some Effects of N^6-benzyladenine on the Morphology and Post-harvest Physiology of Radish (*Raphanus sativus* L.) and Cabbage (*Brassica oleracea* L. var. *capitata* L.)" submitted by Masaru James Tsujita, B. Sc., in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

A majority of the reports presently available concerning the physiological effects of kinins deal with studies of explants. Since the reports on the effects of kinin on intact plants are few, the experiments in Part I of this thesis were conducted to determine the physiological and morphological effects of one kinin, N^6-benzyladenine on intact radish plants.

It was found that high concentrations of N^6-benzyladenine (200 - 400 ppm foliar spray) retarded growth of the radish plants, caused formation of atypical leaves, and inhibited hypocotyl expansion. Lower concentrations of N^6-benzyladenine retarded the development of the radish roots.

In the studies described in Part II of the thesis it was found that N^6-benzyladenine was effective in prolonging storage and maintaining chlorophyll level of cabbages and bunched radishes stored under less than ideal conditions. Higher levels of chlorophyll and RNA were present in kinin-treated cabbages.

Experiments with bunched radishes indicated that kinins may affect a temperature-sensitive system, possibly an enzyme system.

Since evidence to date indicates that kinins appear to be involved in RNA metabolism, Part III of the thesis deals with *in vitro* studies made to determine the effects of kinetin (6-furfurylaminopurine) on RNase degradation of yeast s-RNA. These studies indicated that kinetin inhibits the s-RNA degradation by RNase. It is postulated

that kinins, in some way, may protect the RNA molecule from RNase attack. This may result in retention of protein and chlorophyll levels in kinin-treated explants and thereby delay senescence.

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INTRODUCTION

Kinins, the most recently discovered naturally-occurring growth substances, are known to accelerate cell division (7, 36, 73), induce seed germination (59, 37), and influence leaf growth (30, 52, 53). They have also been found to retard senescence of explants by affecting protein metabolism (47, 54, 66), nucleic acid metabolism (47, 57, 69), and chlorophyll metabolism (5, 49, 67, 50).

Recent reports indicate that one synthetic kinin, N^6-benzyladenine, may be useful in delaying post-harvest deterioration of some species of green vegetables (3, 9, 27, 75, 79). In some investigations N^6-benzyladenine is reported to decrease respiration, whereas in others acceleration in respiration is reported (10, 31, 77).

Information on the effects of kinins on intact plants is meagre. Further studies in this field might prove to be of considerable importance. Therefore, investigations on the effect of N^6-benzyladenine on the growth and development of the radish were initiated and are herein reported.

The retension of green color in vegetables treated with kinins would also be of considerable economic importance in storage and marketing of vegetables in Alberta. For example, the deterioration of chlorophyll during storage is one of the determining factors in consumer acceptance of cabbages. Part II of this thesis pertains to studies conducted to assess the effects of N^6-benzyladenine in the post-harvest retention of quality of cabbage, spinach, and radish.

Since the precise mechanism by which kinins retard senescence still requires elucidation, attempts were also made to determine the probable role of kinins in some biochemical systems. The results of such attempts are outlined in Part III.

REVIEW OF LITERATURE

Purines are fused heterocyclic ring systems which consist of a six-membered pyrimidine ring and a five-membered imidazole ring. They are involved in many important biochemical processes such as energy transfer systems, redox enzyme systems, and genetic coding systems. In 1954, a growth substance which possessed a unique ability to accelerate cytokinesis or cell division was discovered in plant materials (36). Many plant extracts have since been reported to contain a kinetin-like substance (15, 39, 44, 63).

Kinetin and related synthetic compounds have since been found to have physiological and biochemical effects on plants other than acceleration of cytokinesis. A thorough discussion of some of these effects have been presented by Strong (65) and Miller (38) in their reviews. The review of literature in this manuscript, therefore, will be limited to those investigations related more specifically to the studies herein described.

For the purpose of clarifying the terminology used throughout this thesis the term "kinetin" will refer to 6-furfurylaminopurine. The term "kinin" or more appropriately "phytokinin" has been given to a class of kinetin-like compounds which are effective in regulating growth of plants. Therefore, all kinetin-like substances, including kinetin (6-furfurylaminopurine) and N^6-benzyladenine will be classified as kinins.

A. THE EFFECTS OF KININS ON THE GROWTH OF PLANTS

1. Root Growth and Development

The growth-promoting effect of kinin on roots is at present somewhat controversial. Miller (38) reported increased rooting of tobacco stem callus tissue when grown in agar medium containing 0.02 ppm kinetin, casein hydrolysate and indoleacetic acid (IAA). Higher concentrations of kinetin were found to be inhibitory. Fries (14) found increased lateral-root growth of intact seedlings of Lupines hartwegii grown in the absence of light in a medium containing 3×10^{-7} M kinetin. However, Wittwer and Dedolph (75) found that root growth of cucumber and tomato plants grown in culture solution was markedly inhibited as concentrations of kinetin and N^6-benzyladenine were increased. Kemp, Fuller and Davidson also reported on the inhibiting effects of kinetin on root growth of intact tomato plants (28). Arora, Skoog, and Allen (1) were the first to observe and anatomically study "pseudonodules" of tobacco roots grown in 0.2 ppm kinetin. Wittwer and Dedolph also noted similar nodules on roots of cucumber and tomato plants grown in culture solutions containing high concentrations of kinetin and N^6-benzyladenine (28).

2. Leaf Growth and Development

The enlargement of leaf discs of many species of plants floated in kinetin solution has been reported (30, 56). A depression

of primary leaf expansion was observed by Humphries in dwarf bean plants (23). A reduction in top growth of cucumber, tomato, and pea was found by Wittwer and Dedolph when plants were grown in solution culture containing kinetin and N^6-benzyladenine (76). These workers found N^6-benzyladenine to be a more effective kinin than kinetin in suppressing plant growth.

3. Floral Development

Although the effects of kinins on flowering are as yet indefinite, the findings reported to date are of considerable interest. Kemp, et. al. (28) and Wittwer and Dedolph (76) have reported suppression of flowering in tomato. However, Wittwer and Dedolph found kinetin (10^{-8} to 10^{-5} M) to accelerate flowering in "Alaska" variety of peas.

B. THE EFFECTS OF KININS ON THE POST-HARVEST PHYSIOLOGY OF PLANTS

1. Inhibition of Senescence

During the past six years, several chemicals, including certain growth substances, have been found to retard senescence of detached leaf blades. Person, Samborski, and Forsyth (51) found that leaf blades floated on a solution of benzimidazole at a concentration of 50 ppm exhibited a retarded rate of chlorophyll degradation and protein loss. It has also been demonstrated by a number of workers that auxins and gibberellic acid will delay natural yellowing of intact and detached leaves of several species of plants (4, 5, 45, 46).

The kinins have been found to be effective in retarding the degradation of chlorophyll and protein in detached portions

of plants (47, 54). One kinin, N^6-benzyladenine (N^6-benzylaminopurine), was found to be a very effective "senescence inhibitor" in some species of green vegetables (75, 79). Kaufman and Ringel (27) prolonged the marketability of freshly harvested cauliflower heads for about 18 days by applying 10 ppm N^6-benzyladenine or a combination of N^6-benzyladenine and 50 ppm of 2,4-dichlorophenoxy acetic acid. The combination of these chemicals prevented abscission and retarded yellowing of cauliflower heads held at 38° F for 28 days. Zink (79) found that N^6-benzyladenine was also an effective "senescence inhibitor" on lettuce, endive, escarole, Brussels sprouts, radish tops, celery, parsley, green onions, and asparagus. The effective concentration dosage varied with the crop, but 2.5 - 10 ppm at 75 - 100 gallons water per acre gave best results. Preharvest application of N^6-benzyladenine to lettuce at 5 - 10 ppm/100 gallons of H_2O per acre was only effective when made three to four days prior to harvest, indicating that the effects of this substance may be nullified under field conditions. Artichoke, snap beans, and Lima beans did not respond to kinin treatments.

2. Respiration Retarding Effects

Wittwer and Dedolph (77) first reported that N^6-benzladenine appeared to be a respiration retardant in several vegetables. A decreased rate of respiration resulted in retention of freshness in vegetables as a result of smaller loss of weight and green color. Effective response was obtained with 1 - 10 ppm. At 10 ppm N^6-benzyladenine concentration, reductions in respiration rates of asparagus, broccoli, and celery held at 70° F were 16%, 20%, and

27%, respectively (75). Sweet corn, snap beans, and summer squash did not respond to treatments. Dedolph, MacLean, and Wittwer gave further evidence for N^6-benzyladenine being a respiration retardant in some species of vegetables and suggested that it might be involved in phosphate metabolism (8). Smock, Martin, and Padfield studied the effects of N^6-benzyladenine on respiration and storage quality of apples. The kinin treatment was found to accelerate respiration in the preclimateric phase. Since there was a stimulatory effect on respiration in the preclimacteric phase, these authors concluded that it was doubtful whether beneficial effects accrued from applying N^6-benzyladenine to apples in storage.

Lipton and Ceponis (31) found that although senescence was retarded, oxygen consumption in head lettuce was stimulated by application of N^6-benzyladenine at 5, 10, and 20 ppm. They suggested respiration rates higher than normal were consistent with retention of cell vigor and retention of protein in kinin treated leaves, and that morphological and physiological differences among plant parts could be attributed to the decrease in respiration found by other workers.

C. BIOCHEMICAL EFFECTS OF KININS

The mode of action of kinins in influencing cell division, plant growth, respiration, and senescence has not as yet been fully elucidated. Guttman (17) found that kinetin induced a rapid increase in the level of ribonucleic acid (RNA) in the nucleus of onion roots. In Miller's review (38) it is reported that kinetin increased the levels of deoxyribonucleic acid (DNA) and RNA but

did not affect the protein level in onion root cells. Walker and Dietrich (73), working with Tradescantia paludosa, concluded that kinetin accelerated the prophase stage beyond pachytene and that during this stage RNA synthesis reaches a peak. Further enhancement of RNA synthesis by kinetin may be responsible for the acceleration.

Engebrecht and Mothes (13) suggested that inhibition of regeneration and growth of roots of leaf-cuttings may be attributed to the fact that kinetin inhibits the migration of amino acids and other substances from the leaf blade.

Reports on the effectiveness of kinins in maintaining the levels of chlorophyll, protein, and RNA of detached leaves and stems are numerous. Thimann and Laloraya (70) found that buds on pea stem sections grown in IAA and kinetin contained three times as much protein nitrogen as did the controls. The action of the kinetin was found to be localized mainly in the area of application. It is suggested that kinetin may become incorporated into a biochemically active nucleic acid. Sugiura, Umemura, and Oota (66) reported that kinetin at 10^{-5} M suppressed the drop in the protein level of tobacco leaf disks (Nicotians tobacum and N. rustica). The changes in nucleic acid always paralleled those of protein level; this is in agreement with results obtained by Osborne (47). Sugiura, et. al. also found that kinetin induced accumulation of nucleic acid in both the microsomal and supernatant fractions. They concluded that kinetin participatesin protein synthesis through accelerated ribonucleic acid synthesis and that sugar is a prerequisite to the RNA synthesis. The addition of sucrose

to kinetin had a synergistic effect in increasing the levels of nucleic acid and proteins. Osborne (47) came to similar conclusions working with Xanthium leaves and leaf discs. She reported that the incorporation of 14 - C - leucine into protein 14 - C - orotic acid into RNA was increased thus indicating a stimulation of RNA and protein synthesis. However, Shannon and Key (51) found that auxin-induced incorporation of C^{14} nucleotide into RNA was partially inhibited with kinetin concentration of 5 to 10 ug/ml and kinetin at 30 ug/ml eliminated the net increase of RNA. They state that the action of kinetin is comparable to that of inhibitors of RNA synthesis such as 5-fluorouracil and 8-azaguanine. Richmond and Lang (54) suggest that the reduction in loss of chlorophyll in Xanthium leaves may be attributed to a reduction in the rate of loss of protein.

In studies carried out with bean hypocotyls (23), kinetin had no effect on total phosphorus and orthophosphate. However, higher concentrations increased acid-soluble phosphorus, and kinetin at 1 mg/l gave a slight increase in RNA phosphate. Conversely, napthaleneacetic acid increased the levels of all these phosphate compounds.

Yamamoto and Chyama (78) found that kinetin increased the oxidized form of endogenous pyridine nucleotides in the leaves of spinach and tobacco. This suggests that kinetin enhances the glucose utilization by promoting activity of the Embden-Mayerhof pathway, the Pentose phosphate cycle, and the Tricarboxylic acid cycle in the leaves.

Studies on the influence of kinins on the enzymic systems of plants are limited, and precise information is meagre concerning what role or roles they may have in metabolism. It is postulated that one mechanism by which kinins influence the biological system is their regulation of the xanthine oxidase enzyme system as probable substrates or inhibitors (20). Xanthine oxidase was found to oxidize a variety of 6-alkylaminopurines but not 6 alkylthio, 6 alkyloxy, and 6 dialkyl aminopurine derivatives. Maciejewska-Potapczyk (33) reported that "nuclease activity" of bean hypocotyl extract was increased when kinetin was added to the extract. In the presence of IAA the increase was greater than when kinetin alone was present.

In studying the metabolism of benzyladenine by senescing leaves of Xanthium pensylvanicum, McCalla, Morre, and Osborne (35) found that the kinin was converted to a number of low-molecular-weight materials, the major product being the riboside, benzyladenosine. Small amounts of labelled adenylic, guanylic, and inosinic acids, and small amounts of adenine and guanine were produced from 8 - 14 - C benzyladenine. Very little labelled adenylic and guanylic acid was incorporated into the RNA of the Xanthium leaves, nor was benzyladenylic acid incorporated appreciably. A specific biochemical role for kinins could not be suggested by the authors.

PART ONE

I. THE EFFECTS OF N^6-BENZYLADENINE ON THE GROWTH AND DEVELOPMENT OF RADISH (*Raphanus sativus* L.)

The majority of the experiments relating to the effects of kinins on leaf and root growth have been done on explants (13, 18, 25, 52, 53, 56, 71). Reports on the effects of kinins on intact plants are few (28, 76).

In order to further elucidate the effects of kinins on intact plants, experiments were conducted in the greenhouse during the winter months of 1962 - 1963.

Materials and Methods

The common radish, *Raphanus sativus* L., was selected as the plant material because of its short life cycle and ease of culture.

The kinin used in these experiments was a synthetic product of Shell Chemical Company known as Verdan, or more specifically, N^6-benzylaminopurine or N^6-benzyladenine. Verdan is formulated with a water-miscible solvent containing 0.79% (by weight) active ingredients. In these studies the kinin was applied as a foliar spray treatment.

The experimental design was a randomized block design with five treatments replicated three times. Each treatment consisted of eight 6-inch clay pots with 5 plants per pot for a total of 40 plants. Guard rows were used along the edge of the

greenhouse bench. The five treatments are listed in Table I.

Seeds of the Early Scarlet Globe radish were sized for uniformity and planted in 6-inch clay pots containing a soil mixture of 3 parts soil, 1 part sand and 2 parts peat. When the radish plants had grown to the first-true-leaf stage, they were thinned out to five plants per pot. A week later when the first true leaf had fully expanded, the first foliar application of N^6-benzyladenine was applied with a fine-spray cosmetic atomizer. A second application was made a day later with the same concentration for each treatment.

The following observations were made:

1. Number of expanded hypocotyls 3 weeks after treatment.
2. Number of radishes of marketable size 4 weeks after treatment.
3. Measurements of leaf width and length 4 weeks after treatment.
4. Weight of leaves and roots of a random sample of 2 plants from a pot harvested from each treatment.
5. Length of flower stalks of the 2 plants per pot left to mature.

An effort was also made to determine the toxic levels and study the morphological effects of high concentrations of this kinin. A randomized block design was used, with four treatments replicated four times. Seven pots were used for each treatment, each pot containing 2 plants, giving a total of 14 plants per treatment. The concentrations used were as follows:

Treatments	N^6-benzyladenine Conc.
1	0 ppm
2	100 ppm
3	200 ppm
4	400 ppm

Results

1. General

No gross effects due to the kinin treatment were noticed a day following the first spray treatment. However, a week after the second spray application the leaves of plants in the fifth treatment (160 ppm) began to show slight signs of chlorosis with purplish pigment accumulation around the edges. The third and fourth treatments (200 ppm and 400 ppm) showed chlorotic effects and a backward cupping of the young leaves (Illustration # 1). At 200 ppm the crown region of the hypocotyl became expanded and swollen within one week. The major portion of the hypocotyl failed to expand although the epidermal layer broke (Illustration # 2). A callus growth developed from these broken regions from which a mass of root-like growths was initiated (Illustration # 3).

2. Root Growth and Development

The application of kinin significantly delayed the expansion of the hypocotyl as can be seen in Table I. Duncan's Multiple Range Test (12) indicated that plants sprayed with 20, 40, 80, and 160 ppm N^6-benzyladenine had significantly less expanded hypocotyls than the control plants but there were no significant differences among the treatments.

Illustration 1 The effects of N^6-benzyladenine on leaf growth of radish. The plants on the left treated with 200 ppm as foliar spray at first true-leaf stage. Control plants on the right.

Illustration 2 The effects of N^6-benzyladenine on root growth of radish. The upper plant treated with 200 ppm at first true-leaf stage as a foliar spray. Control plant on the bottom.

Illustration 3 A magnified portion of the radish hypocotyl showing callus growth. Note root-like structures protruding from the callus.

TABLE I

A. Mean numbers of expanded radish hypocotyls three weeks after treatment with N^6-benzyladenine

Treatment	Mean numbers
0 ppm N^6-benzyladenine	30.6
20 ppm N^6-benzyladenine	22.6*
40 ppm N^6-benzyladenine	22.3*
80 ppm N^6-benzyladenine	21.3*
160 ppm N^6-benzyladenine	20.3*

B. Analysis of variance of total number of expanded hypocotyls

Source of variation	d.f.	SS	MS	F
Replication	2	20.9		
Treatments	4	204.4	51.1	4.73*
Error	8	86.4	10.8	
F.05 (4, 8) = 3.84			F.01 (4, 8) = 7.01	

* Exceeds the .05 level of significance.

TABLE II

A. Mean numbers of marketable size radish four weeks after foliar treatment with N^6-benzyladenine

Treatment	Mean numbers
0 ppm N^6-benzyladenine	13.33
20 ppm N^6-benzyladenine	7.00*
40 ppm N^6-benzyladenine	3.00**
80 ppm N^6-benzyladenine	3.33**
160 ppm N^6-benzyladenine	8.33*

B. Analysis of variance of total number of marketable size radish

Source of variation	d.f.	SS	MS	F
Replication	2	26.8		
Treatments	4	136.4	34.1	7.3**
Error	8	37.2	4.7	

F.05 (4, 8) = 3.84 F.01 (4, 8) = 7.01

* Exceeds the .05 level of significance.

** Exceeds the .01 level of significance.

TABLE III

A. Mean weights in grams of radish roots harvested

Treatment	Mean Weights
0 ppm N^6-benzyladenine	72.00
20 ppm N^6-benzyladenine	57.83
40 ppm N^6-benzyladenine	54.83*
80 ppm N^6-benzyladenine	47.33*
160 ppm N^6-benzyladenine	58.00

B. Analysis of variance of total root weight

Source of variation	d.f.	SS	MS	F
Replications	2	324.90		
Treatments	4	957.50	239.37	4.43*
Error	8	432.60	54.07	

$F_{.05}$ (4, 8) = 3.84

* Exceeds the .05 level of significance.

The development of the hypocotyl from hypocotyl expansion to marketable stage was influenced to a significant degree, as is shown by the figures in Table II. The Multiple Range Test of the treatment means revealed that N^6-benzyladenine at 20 and 160 ppm significantly reduced hypocotyl development, and, at concentrations of 40 and 80 ppm reduction in hypocotyl development was highly significant.

The total fresh weight of 16 radish taproots was recorded at harvest for each treatment. Analysis of variance in Table III indicates significant differences among the spray treatments. Duncan's Multiple Range Test shows that N^6-benzyladenine at concentrations of 40 ppm and 80 ppm significantly reduced the radish yield. The kinin concentration of 20 ppm and 160 ppm did not significantly reduce the yield.

3. Leaf Growth and Development

The longest leaf on each of the four plants in a pot was measured for maximum width and length five days prior to harvest. Although the mean width and length of the treated plants showed slight increases, these were not statistically significant (Table IV and V).

The leaves of plants used in measuring root weights were retained and the total leaf weights were recorded. There were no significant differences among any of the treatments, including the control (Table VI).

4. Flower Development

The studies on the effects of N^6-benzyladenine on flowering were complicated in this experiment by the fact that the radish is a long-day plant. Although artificial light was employed to extend daylength from 5 - 8 P.M., the growth of the radish was extremely slow. In conjunction with the addition of supplemental light the temperature was raised from 65° to 75° F to induce bolting. The analysis of variance of the measurements taken of the flower stalks after three months' growth showed no difference in the treatments (Table VII).

Discussion

The results obtained from the experiments involving spraying of radishes indicated that N^6-benzyladenine influences the growth and development of the hypocotyl. A retardation in growth and development was brought about by 20, 40, 80, and 160 ppm concentrations. Although the method of application differed in these experiments, the results agree with those of Wittwer and Dedolph (76) on cucumber, tomato, and pea, and Kemp *et al.* (28) on tomato. Concentrations of 200 ppm not only inhibited hypocotyl expansion but caused formation of callus-like growths or pseudo-nodules of the kind observed by others (1, 55, 76) in tomato, tobacco, and cucumber roots.

A possible explanation for the retardation in root growth may be found in the hypothesis of Engebrecht and Mothes (13) who suggested that the inhibition of the regeneration and growth

TABLE IV

A. Mean widthsof radish leaves

Treatment	Mean widths in cm.
0 ppm N^6-benzyladenine	1.93
20 ppm N^6-benzyladenine	2.03
40 ppm N^6-benzyladenine	2.00
80 ppm N^6-benzyladenine	2.03
160 ppm N^6-benzyladenine	2.03

B. Analysis of variance of mean leaf widths

Source of variation	d.f.	SS	MS	F
Replications	2	.001		
Treatments	4	.023	.0058	1.76
Error	8	.026	.0033	

F.05 (4, 8) = 3.84

TABLE V

A. Mean lengths of radish leaves

Treatment	Mean lengths in cm.
0 ppm N^6-benzyladenine	6.3
20 ppm N^6-benzyladenine	6.5
40 ppm N^6-benzyladenine	6.3
80 ppm N^6-benzyladenine	6.4
160 ppm N^6-benzyladenine	6.5

B. Analysis of variance of mean leaf lengths

Source of variation	d.f.	SS	MS	F
Replications	2	.13		
Treatments	4	.23	.058	n.s.
Error	8	.87	.109	

TABLE VI

A. Mean weights of radish leaves

Treatment	Mean weights in gm.
0 ppm N^6-benzyladenine	97.66
20 ppm N^6-benzyladenine	96.66
40 ppm N^6-benzyladenine	93.00
80 ppm N^6-benzyladenine	100.33
160 ppm N^6-benzyladenine	106.66

B. Analysis of variance of mean leaf weights

Source of variation	d.f.	SS	MS	F
Replications	2	311		
Treatments	4	316	79	n.s.
Error	8	993	124	

TABLE VII

A. Mean heights of radish seed-stalk in cm.

Treatment	Mean heights in cm.
0 ppm N^6-benzyladenine	3.92
20 ppm N^6-benzyladenine	3.55
40 ppm N^6-benzyladenine	3.82
80 ppm N^6-benzyladenine	3.24
160 ppm N^6-benzyladenine	4.84

B. Analysis of variance of the mean seed-stalk heights

Source of variation	d.f.	SS	MS	F
Replications	2	5.48		
Treatments	4	5.28	1.32	1.4
Error	8	7.13	.89	

$F_{.05}$ (4, 8) = 3.84

of roots from leaf cuttings may be attributed to kinin inhibition of nitrogenous-material transport from the leaf blades to the rooting area. They have reported that nitrogenous materials (amino acids, etc.) migrate to the kinin-treated area and accumulate in this region (40, 42).

Only high concentrations of spray (above 160 ppm) of N^6-benzyladenine showed any effects on leaf growth and development. No increase in the rate of growth was observed at lower rates and concentrations above 160 ppm caused stunting of the plants and abnormal leaf growth, as reported by others (28, 38). Signs of chlorosis and an accumulation of a purplish pigment along the edges of the leaves were observed. (Similar observations are discussed in Miller's review (38) where an increase of anthocyanin in cultured petals of Impatiens balsamina is reported. Bamberger and Mayer (2) found that an increase in red pigment occurred in Amaranthus retroflexus seedlings treated with kinetin). At 200 ppm the lobes of the radish leaves failed to develop and unusually long petioles were prominent. A backward cupping of the leaves occurred, indicated that a differential rate of cell division or of elongation may have been induced by the kinin. The chlorosis and abnormalities of leaf shape gradually disappeared as the plants apparently overcame the physiological and cytological effects of kinin. Possibly the plants may have been able to metabolize kinin, which, since spraying was limited to two applications, may thus have been completely used up. At the time of harvest no significant differences were found in leaf

width, leaf length, or fresh weight of leaves.

The application of N^6-benzyladenine at the first-true-leaf stage did not appear to influence the development of flowers or the elongation of the flower stalks. The results shown in Table VII indicate that great variability existed in the development of the flower stalks.

PART TWO

II. THE EFFECTS OF N^6-BENZYLADENINE ON THE STORAGE LIFE AND QUALITY OF SOME SPECIES OF VEGETABLE CROPS

The role of N^6-benzyladenine as a respiration retardant and senescence inhibitor in certain vegetable crops is promising. This and other kinins have been shown to prolong the fresh quality of vegetables throughout the transit and storage phases in some crops (3, 9, 27, 31, 77). These substances may therefore be of great benefit to producers and marketing agents.

The effects of kinins on the storage of root vegetables have not yet been reported, and, as a consequence, investigations dealing with the effect of N^6-benzyladenine on radishes were carried out.

The possibility of kinins affecting the retention of green color would also be of great economical importance in the storage and marketing of spinach and cabbage in Alberta. Deterioration of the chlorophyll content of late varieties of cabbage stored during the winter is a determining factor in consumer acceptance of Alberta-grown cabbages. Therefore, special emphasis was given to the studies of the effects of N^6-benzyladenine on chlorophyll-retention and quality of stored cabbages.

A. STUDIES ON THE EFFECTS OF N^6-BENZYLADENINE ON THE STORAGE AND QUALITY OF RADISHES (*Raphanus sativus* L.)

1. Bunched Radish Experiment

Materials and Methods

Early Scarlet Globe radishes grown in the greenhouse were harvested and bunched into groups of ten plants. The treatments consisted of dipping the bunches (leaf and root) in an aqueous solution of 0, 20, or 40 ppm N^6-benzyladenine for five minutes. Each bunch was then placed in a polyethylene bag (2 mil) with a uniform number of holes punched for aeration. Nine bags representing three treatments replicated three times, were stored at 36° ± 2° F and six bags, representing three treatments replicated twice, were stored at 46° ± 2° F.

Results

One replicate of each treatment was removed after 8 days storage at 36° ± 2° F. The radishes in all these replicates were in excellent condition. Upon storing these for four days in the dark room temperature (approx. 70° F) differences among treatments became evident, indicating that the temperature had a pronounced effect on the action of N^6-benzyladenine (Illustration 4 and 5). The results led to the hypothesis that an enzyme system may be implicated in the control of senescence. (The studies and results of the influence of kinetin on ribonuclease and possible enzymic control of senescence by kinins are discussed in detail in the latter part of this thesis.)

Illustration 4 The effects of 20 and 40 ppm concentrations of N^6-benzyladenine on bunched radishes stored at 36° ± 2° F for 12 days. Control bunches on the left.

Illustration 5 The effects of 20 and 40 ppm concentrations of N^6-benzyladenine on radishes stored at 36° ± 2° F for 8 days then at 70° F for an additional 4 days. Control bunch on the left.

Of the bunches stored at 36° ± 2° F for 55 days, those treated with kinin at 20 and 40 ppm retained the green color and fresh quality, while leaves of the control plants had become mottled with yellow.

At 46° ± 2° F, those receiving 20 ppm treatment showed a slight yellowing after 25 days and those receiving 40 ppm remained green. The application of 20 and 40 ppm N^6-benzyladenine prolonged marketability approximately one week. The control bunches stored at 46° ± 2° F for 25 days turned completed yellow.

2. Root Storage Experiment

Materials and Methods

Field grown Cherry Belle radishes were harvested July 11 and sized into three groups. Radishes were selected and pooled from each group until 18 representative samples weighing 410 ± 2 grams were obtained. These were placed in a 2 mil polyethylene bag which had equal numbers of holes punched for aeration. The contents of the nine bags were soaked for two minutes in solutions containing 0, 50, and 100 ppm N^6-benzyladenine and returned to the bags. The nine bags, representing three replications of the treatments were stored at 36° ± 2° F. Another nine bags treated in a similar manner were stored at 46° ± 2° F. The weight of each bag was recorded at two-week intervals for a total of ten weeks.

Results

The effectiveness of N^6-benzyladenine as a respiration retardant did not apply to radish roots stored at 36° ± 2° F, or at 46° ± 2° F, (Tables VIII and IX). Analysis of variance of loss of weight during ten weeks storage at 36° ± 2° F and 46° ± 2° F showed no significant difference in the effects of the various treatments. However, during the period between the eighth and tenth week, N^6-benzyladenine-treated radishes lost somewhat less weight than the control radishes at both temperatures.

B. STUDIES ON THE POST-HARVEST TREATMENT OF SPINACH (*Spinacia oleracea* L.) WITH N^6-BENZYLADENINE

Materials and Methods

The Bloomsdale Long Standing Dark Green variety of spinach, grown in the greenhouse, was harvested and dipped into concentrations of 0, 10, and 20 ppm N^6-benzyladenine. The spinach plants were divided into groups of four plants, one group for each treatment. After treatment each group was placed in 2 mil polyethylene bags with a uniform number of holes. Each treatment was replicated four times and stored at 36° ± 2° F, 46° ± 2° F, 60° ± 2° F and room temperature (70° F).

Results

The treated spinach stored at room temperature deteriorated within four days and treatment effects were not visible.

TABLE VIII

A. Mean weight loss in grams of radish roots stored at 36° ± 2° F treated with 0, 50, and 100 ppm N^6-benzyladenine

	Length of storage in weeks				
Treatment	2	4	6	8	10
0 ppm	1.06	8.16	22.00	35.00	42.53
50 ppm	.30	9.00	20.53	25.83	32.13
100 ppm	.76	9.16	14.83	27.33	36.06

B. Analysis of variance of weight loss during 10 weeks storage at 36° ± 2° F

Source of variation	d.f.	SS	MS	F
Replications	2	14.2		
Treatments	2	178.1	89.05	5.5 n.s.
Error	4	64.2	16.05	
F.05 = 6.94				F.01 = 18.00

TABLE IX

A. Mean weight loss in grams of radish roots stored at 46° ± 2° F treated with 0, 50, and 100 ppm N^6-benzyladenine

	Length of storage in weeks				
Treatment	2	4	6	8	10
0 ppm	1.16	10.16	17.06	29.30	47.03
50 ppm	2.16	10.33	19.60	29.10	37.76
100 ppm	1.56	9.83	19.16	25.30	42.03

B. Analysis of variance of weight loss during 10 weeks storage at 46° ± 2° F

Source of variation	d.f.	SS	MS	F
Replications	2	20		
Treatments	2	193	96.5	5.2 n.s.
Error	4	74	18.5	
F.05 = 6.94				F.01 = 18.00

When stored at 60° ± 2° F, there was slight deterioration after 5 days and complete deterioration was observed by seven days. There was some indication that the treatment had a beneficial effect with spinach stored at 46° ± 2° F for nine days. The treated spinach showed less yellowing than the control. The spinach lots stored at 36° ± 2° F remained green after twelve days. The effectiveness of N^6-benzyladenine on spinach stored at unfavorable storage temperatures could not readily be assessed owing to the rapid deterioration of the spinach at these temperatures. Because of its unfavorable response to storage, no further studies with spinach were made.

C. STUDIES ON THE EFFECTS OF N^6-BENZYLADENINE ON THE STORAGE AND QUALITY OF CABBAGES (*Brassica oleracea* L. var. capitata L.)

Five varieties of field grown cabbages were used in these studies; Early Marvel, Glory of Enkhuizen, Jumbo, Pennstate Ballhead, and Danish Ballhead. The first four varieties were started from seed in the greenhouse and the seedlings were transplanted into field plots in the spring. The Danish Ballhead variety was grown from direct seeding in the field. Oliver Institute supplied the Pennstate variety for the experiment conducted in their root cellar.

1. Chlorophyll Content of Early Marvel Cabbage

One of the criteria that may be used to evaluate the quality of green vegetables is chlorophyll content. An experiment was conducted to ascertain if chlorophyll levels could be used as an index of quality for cabbages treated with N^6-benzyladenine.

Materials and Methods

Early Marvel cabbages were harvested on July 22 and immediately dipped in solutions containing 0, 10, 20, and 30 ppm N^6-benzyladenine. Two ml of Tween-20 per gallon of solution were used as a spreading agent. The experiment consisted of a total of 24 uniform heads of cabbages. Each treatment was applied to two heads and there were three replications. These lots of cabbages were stored at 40° ± 1° F until September 6 (45 days) and then sampled for chlorophyll determination.

Samples were taken with a cork borer (1 cm diameter). Ten plugs approximately 1 cm in depth were taken at random from each head. The twenty plugs from each treatment were then thoroughly mixed and three two-gram samples were weighed out to provide three determinations per sample.

The two-gram samples were ground in a mortar and chlorophyll was extracted with 100% reagent-grade acetone. The acetone solution was filtered through two layers of Whatman's No. 1 filter paper and made up to 50 ml volume. Three mls of the acetone solution containing extracted chlorophyll was pipetted into a 1 cm sq. quartz cuvette and the absorbancies were determined at wavelengths of 645 and 663 mu. The absorbancy readings from the three determinations were averaged to obtain the treatment absorbancy value. These average values were then converted to mg chlorophyll per gram fresh weight by the use of a conversion formula (34), as follows:

Chlorophyll a = (0.01230 D 663 - 0.00086 D 645) V/W

Chlorophyll b = (0.0193 D 645 - 0.0036 D 663) V/W

where, D = density or absorbancy, V = volume,
W = weight of sample.

Results

N^6-benzyladenine effectively retarded yellowing of Early Marvel Cabbage and prolonged marketability two weeks. Visible differences were found after three weeks of storage at 40° ± 1° F. Total chlorophyll (a + b) extracted from cabbages treated with 10 and 20 ppm N^6-benzyladenine was more than twice that extracted from the controls. The contents of cabbages treated with 30 ppm was four times that of the controls (Table X C). The analysis of variance indicated that the differences among the treatments were highly significant. The comparison of treatment means with the use of Duncan's Multiple Range Test (12) revealed that the treatments involving 10 ppm and 20 ppm N^6-benzyladenine were significantly higher in chlorophyll than the control. There were no differences between the 10 ppm and 20 ppm treatment. The increase in chlorophyll content by the 30 ppm treatment was highly significant, even when this treatment was compared with the other three treatments.

TABLE X

THE INFLUENCE OF N^6-BENZYLADENINE ON CHLOROPHYLL LEVELS IN EARLY MARVEL CABBAGE

A. Absorbancy readings of chlorophyll extracts

N^6BA	Rep I		Rep II		Rep III	
Conc.	645 mu	663 mu	645 mu	663 mu	645 mu	663 mu
0 ppm	.045	.053	.022	.035	.035	.047
10 ppm	.063	.130	.065	.130	.063	.093
20 ppm	.053	.133	.060	.098	.067	.117
30 ppm	.145	.265	.085	.163	.102	.197

B. Absorbancy readings converted to mg chlorophyll/gm fresh weight

N^6BA	Rep I Chlorophyll		Rep II Chlorophyll		Rep III Chlorophyll	
Conc.	a	b	a	b	a	b
0 ppm	.0153	.0069	.0102	.0075	.0137	.0126
10 ppm	.0386	.0187	.0303	.0221	.0272	.0220
20 ppm	.0398	.0136	.0288	.0202	.0346	.0218
30 ppm	.0783	.0461	.0483	.0263	.0584	.0312

TABLE X (Continued)

C. Total chlorophyll (a + b)/mg/gram fresh weight

N^6BA Conc.	Rep. I	Rep. II	Rep. III	Mean
0 ppm	.0222	.0177	.0263	.0221
10 ppm	.0573	.0524	.0492	.0527*
20 ppm	.0534	.0490	.0564	.0527*
30 ppm	.1245	.0746	.0896	.0950**

D. Analysis of variance on the level of total chlorophyll content

Source of variation	SS	d.f.	MS	F
Replications	.000421	2		
Treatments	.008095	3	.002698	26.2**
Error	.000719	6	.000103	

F.05 (3, 6) = 4.76 F.01 (3, 6) = 9.78

* Exceeds the .05 level of significance.

** Exceeds the .01 level of significance.

2. Quality and Chlorophyll Levels of Pennstate Ballhead Cabbages Stored under Root Cellar Conditions

In order to ascertain the effectiveness of N^6-benzyladenine in retaining the quality and chlorophyll level of cabbages held in root cellar storage, an experiment was conducted in co-operation with Oliver Institute, four miles east of Edmonton. The effects of N^6-benzyladenine on cabbages that had been harvested and stored for approximately three weeks prior to treatment were also assessed.

Materials and Methods

On October 29, 1963, one hundred uniform heads of Pennstate Ballhead cabbages that had been stored for approximately three weeks at 36 ± 2° F were selected from the storage bin and the outer leaves removed for better uniformity prior to treatment. Tween-20 at a concentration of 0.5 ml per gallon was added to each of the two treatments, which consisted of a control and N^6-benzyladenine at 50 ppm. The experimental design used was a randomized block consisting of five replications. Each replication was made up of 10 treated heads and 10 control heads. Each replication was placed in a separate wooden crate.

On December 2, 1963 (after approximately one month storage), ten leaf plugs were randomly sampled from each of the heads. The 100 plugs were manipulated so as to separate the leaf discs and thoroughly mix them, and each sample was freeze-dried. The weights of the dessicated leaf discs were recorded prior to the extraction of chlorophyll.

The dry leaf discs were ground finely with sand in a mortar using 80% acetone to extract the chlorophyll. The ground material was filtered off using two layers of Watman's No. 1 filter paper. The extracted, filtered solution was made up to 50 ml volume with 80% acetone. Three mls of chlorophyll solution were pipetted into a 1 cm sq. quartz cuvette and absorbancy was read using the Beckman DK spectrophotometer. The absorbancy values at 645 and 663 mu were converted to mg chlorophyll/gram dry weight basis using the same conversion formula as in chlorophyll determination with Early Marvel cabbages.

Results

The treatment of Pennstate Ballhead cabbage with 50 ppm N^6-benzyladenine effectively maintained the chlorophyll at a higher level even when applied approximately three weeks after harvest. Significant differences in the chlorophyll content were indicated by the analysis of variance. Treated cabbages contained higher levels in all replications (Table XI).

TABLE XI

A. Total chlorophyll extracted from Pennstate Ballhead Cabbages (mg/gram dry weight)

N^6BA	Rep I	Rep II	Rep III	Rep IV	Rep V	$\bar{X}$
0 ppm	.045	.070	.065	.080	.075	.067
50 ppm	.080	.105	.150	.180	.195	.142

B. Analysis of variance of total chlorophyll extraction from Pennstate Ballhead cabbages treated with 0 and 50 ppm N^6-benzyladenine

Source of variation	d.f.	SS	MS	F
Replications	4	.007285		
Treatment	1	.014062	.014062	18.90*
Error	4	.002975	.000744	

F.05 (1, 4) = 7.71 F.01 (1, 4) = 21.20

* Exceeds the .05 level of significance.

3. Pre-harvest Spray Treatment on the Storage Life and and Quality of Glory of Enkhuizen Cabbages

A study was conducted to evaluate the senescence-inhibiting properties of N^6-benzyladenine when applied as a pre-harvest foliage spray.

Materials and Methods

Three 30-foot rows of Glory of Enkhuizen cabbage were sprayed with 0, 20, and 40 ppm N^6-benzyladenine respectively. Tween-20 at one ml per gallon was used as a spreader. Five heads from each treatment were harvested immediately after spraying and the remainder were harvested 24 hours after spraying. The cabbages were stored at 40° ± 1° F immediately after harvest.

Results

During the 21 days of the storage period there did not appear to be any visible differences in the cabbages harvested immediately after spraying and those harvested 24 hours later. In all cases the spray treatments with 20 ppm and 40 ppm kinin definitely enhanced the storage life and quality of the treated cabbages over those receiving no treatment. The difference between treated and control cabbages were noticeable in about four weeks. A panel of four judges unanimously agreed that the treated cabbages were superior in color, appeal, and freshness. Cabbages treated with 40 ppm kinin were rated higher than those treated with 20 ppm (Table XII). The marketability of this variety was prolonged 20 days.

TABLE XII

Evaluation of pre-harvested spray-treated Glory of Enkhuizen cabbages by a panel of judges

Judge Number	Color	Appeal	Freshness
1	A, C, B	A, C, B	A, C, B
2	A, C, B	A, C, B	A, C, B
3	A, C, B	A, C, B	A, C, B
4	A, C, B	A, C, B	A, C, B

The treatments were labelled as following:

A = 40 ppm, B = control, C = 20 ppm

The above ranked in order of superiority.

4. Storage Life and Quality of Jumbo, Pennstate Ballhead, and Danish Ballhead Cabbages

Materials and Methods

Jumbo and Pennstate Ballhead cabbages were dip-treated with 0, 30 and 40 ppm N^6-benzyladenine. Danish Ballhead variety was treated with 0 and 50 ppm N^6-benzyladenine. All the experiments on these varieties were conducted at 40° ± 2° F. Three replications were made using ten heads per treatment.

Results

In all the experiments the kinin-treated cabbages were superior in color and quality after approximately six weeks of storage. The three varieties are good storing types and did not show response to kinin until 45 days after treatment. The condition of Jumbo and Pennstate Ballhead varieties stored for 53 and 47 days, respectively, is shown in Illustration 6 and 7.

Illustration 6 The effects of 30 and 40 ppm N^6-benzyladenine dip treatments on Jumbo variety cabbages stored at 40° F for 53 days. Control cabbages on the right.

Illustration 7 The effects of 50 ppm N^6-benzyladenine dip treatment on Pennstate Ballhead variety cabbages stored at 40° F for 47 days. Controls on the right.

Discussion

The application of kinin to extend the shelf life of bunched radishes may be of some advantage under unfavorable storage conditions. Concentrations of 20 - 40 ppm N^6-benzyladenine retained the quality and green color of the leaf portion and extended the marketable life of the bunched radishes by nearly a week at a temperature of 46° ± 2° F. The effectiveness of this kinin on radish tops has also been reported by Zink (79). The response of kinin treatment in the experiments reported here was less pronounced at 36° ± 2° F. However, if the radishes were brought out and stored at room temperature (in darkness) the beneficial effect of the treatment became more pronounced. This suggests a practical use of kinin in prolonging shelf life of bunched radishes during retail marketing.

The temperature-kinin effect observed indicates that N^6-benzyladenine influences certain metabolic mechanisms which increase in activity at temperatures above the ideal storage temperature. The kinin, therefore, may be suspected to have an inhibitory effect on some metabolic process which is accelerated at higher temperatures.

Spinach appeared to be less responsive than the radish to the kinin treatment. Although the rapid deterioration of spinach made evaluation difficult in tests wherein the storage conditions were most ideal and storage lasted more than two weeks, kinin appeared to be of some benefit. The high chlorophyll content of spinach leaves may influence the visual evaluation during short storage periods.

We have observed a lesser degree of response to kinin treatment in spinach and other workers have reported inconsistency in both respiration and senescence response. A probable explanation of these inconsistencies is that incorporation of kinins into the plant tissues may be very important. The physical make-up of the plant tissue may influence the amount of kinin which is taken up in various species. It has been reported that sweet corn, snap beans, summer squash, artichoke, Lima beans and apples in the preclimacteric stage do not respond to kinin treatment (61). The inconsistent effects on respiration response reported by various workers (10, 26, 31, 61, 68, 75, 77) may be explained by the suggestion by Lipton and Ceponis (31) that the degree of retardation of respiration by kinin varies with different plants and plant tissues.

Studies with radish roots (hypocotyls) stored over a period of ten weeks indicated that N^6-benzyladenine is not highly effective in slowing down the respiration process and subsequent weight loss. The weight loss was reduced to some degree but the reduction was not statistically significant.

N^6-benzyladenine was highly effective in maintaining the chlorophyll level and quality of the cabbage. The marketability of cabbages could be extended two to six weeks depending on the variety and the method of application. Kaufman and Ringel (27) reported similar findings with cauliflower. They were able to extend the marketability eighteen to twenty-eight days. Dip treatment appeared to be the most effective method of kinin application probably because it allows a better and more lasting exposure to the kinin.

Zink (79) reported that field-sprayed kinin treatment was only effective on lettuce if the heads were harvested within three days after spraying. Responses to kinin were obtained with cabbages if the heads were harvested within 24 hours after field spray application. N^6-benzyladenine was effective on Pennstate Ballhead cabbages even when dip treatment was applied three weeks after harvest. This suggests that kinin may possibly be used effectively if treatments were applied prior to display for sale in the stores.

The extension of marketability of Early Marvel cabbages was approximately two weeks over the control.

The marketability of superior storage types such as the Pennstate Ballhead, Danish Ballhead, and Jumbo varieties was extended by approximately one month when stored at a temperature of 40° ± 1° F. The most ideal storage temperature for cabbages would be 32° F with 95% relative humidity. However, storage at 40° ± 1° F illustrated the beneficial effects of kinin under less ideal conditions. Therefore, kinins may provide the most practical benefit by lengthening the storage life and quality of some vegetables stored or shipped under less than ideal conditions.

III. THE BIOCHEMICAL ROLE OF KININS IN THE CONTROL OF SENESCENCE

The results obtained from the experiments with radish tops and cabbages support the view that N^6-benzyladenine is an effective senescence inhibitor in some species of green leafy vegetables. However, the mechanism by which kinins affect the biochemical processes leading to the senescence of explants requires further elucidation.

Reports by others (47, 57) indicate that kinins are implicated in the nucleic acid metabolism, but the precise mode of action is yet to be resolved. It is generally accepted that nucleic acids govern the biosynthesis of proteins (74). Osborne (47), suggests that kinetin may maintain the synthesis of ribonucleic acid (RNA) and protein in Xanthium. Sugiura (67) found that kinetin promoted chlorophyll accumulation in excised primary bean leaf during three hours illumination and suggested that kinetin in collaboration with light may accelerate the organization of chlorophyll synthesizing machinery. However, since most vegetable storage experiments with kinins have been conducted in the dark, there must be some other control mechanism. It is highly probable that protein stabilization of the chlorophyll lipoprotein complex may be induced by kinins, thus governing the rate of chlorophyll deterioration.

Shannon and Key (57) found that IAA, at growth-promoting concentrations, enhanced C^{14} nucleotide incorporation into RNA, but kinetin at 5 to 10 ug/ml inhibited the auxin-induced synthesis of RNA. Therefore, another probable and logical alternative

suggestion for the basic mode of action of kinins is that they may in some way inhibit the natural degradation of RNA, and as a consequence maintain protein level in the chloroplast and the cytoplasm.

Supporting evidence for the hypothesis that kinins may inhibit natural degradation of RNA was obtained from experiments in which the response of radish leaves to N^6-benzyladenine was observed. Leaves of bunched radishes treated with 0, 20, and 40 ppm N^6-benzyladenine and stored in the dark at 36° F for a period of 12 days showed no sign of senescence. When radishes receiving the same treatments were stored under the same conditions for 8 days, then removed and held at room temperature (70° F) in absence of light for 4 days, the leaves of control plants turned completely yellow, while the leaves treated with 20 and 40 ppm kinin remained green (Illustrations 4 and 5). These observations suggested that a temperature sensitive mechanism was involved in the maintenance of chlorophylls. An enzyme system would be a possibility. Osborne (47) found that RNA levels were maintained in Xanthium leaf discs, treated with kinetin, that were stored in the dark for 1 - 4 days at 24° C. We have thus hypothesized that the enzyme, ribonuclease (RNase), which degrades RNA by hydrolyzing the nucleic acid polymer into mononucleotides, dinucleotides, and oligonucleotides (64), may be involved.

In an attempt to further explain the mechanism by which kinins control senescence, <u>in vitro</u> studies were initiated to determine the effects of one kinin, kinetin (6-furfurylaminopurine), on RNase activity.

In addition, the RNA and chlorophyll levels of N^6-benzyladenine-treated cabbages were determined to ascertain whether the levels of these materials were maintained by kinin treatments as reported in the work with Xanthium (47, 49) and tobacco (67, 68).

A. IN VITRO STUDIES ON THE EFFECT OF KINETIN ON RIBONUCLEASE ACTIVITY

Materials and Methods

Ribonuclease (RNase), 5X crystalline, and yeast ribonucleic acid (RNA) were obtained from Nutritional Biochemical Corporation. The soluble yeast RNA was purchased from General Biochemicals and kinetin (6-furfurylaminopurine), grade A, was obtained from California Corporation for Biochemical Research. For all reactions and measurements, RNase, RNA, and kinetin were dissolved in a 0.1 M acetate buffer, pH 5.

Two spectrophotometric methods of assay were used to study the activity of RNase on the substrate, RNA. The first method was a modification of that of Kunitz (29), in that the absorption increase at 260 mu, rather than at 300 mu, was taken as a measurement of enzyme activity. Kunitz's method is based on the fact that digestion of RNA by RNase causes a diminution in ultraviolet absorption by RNA and a gradual shift in the ultraviolet absorption spectrum in a region of 290 to 305 mu (Fig. 1). This method was considered inadequate since the shift and absorption decease at 300 mu was very small. Therefore, readings were taken in the 260 mu region, where the maximum increase in absorption was observed upon digestion of RNA with RNase.

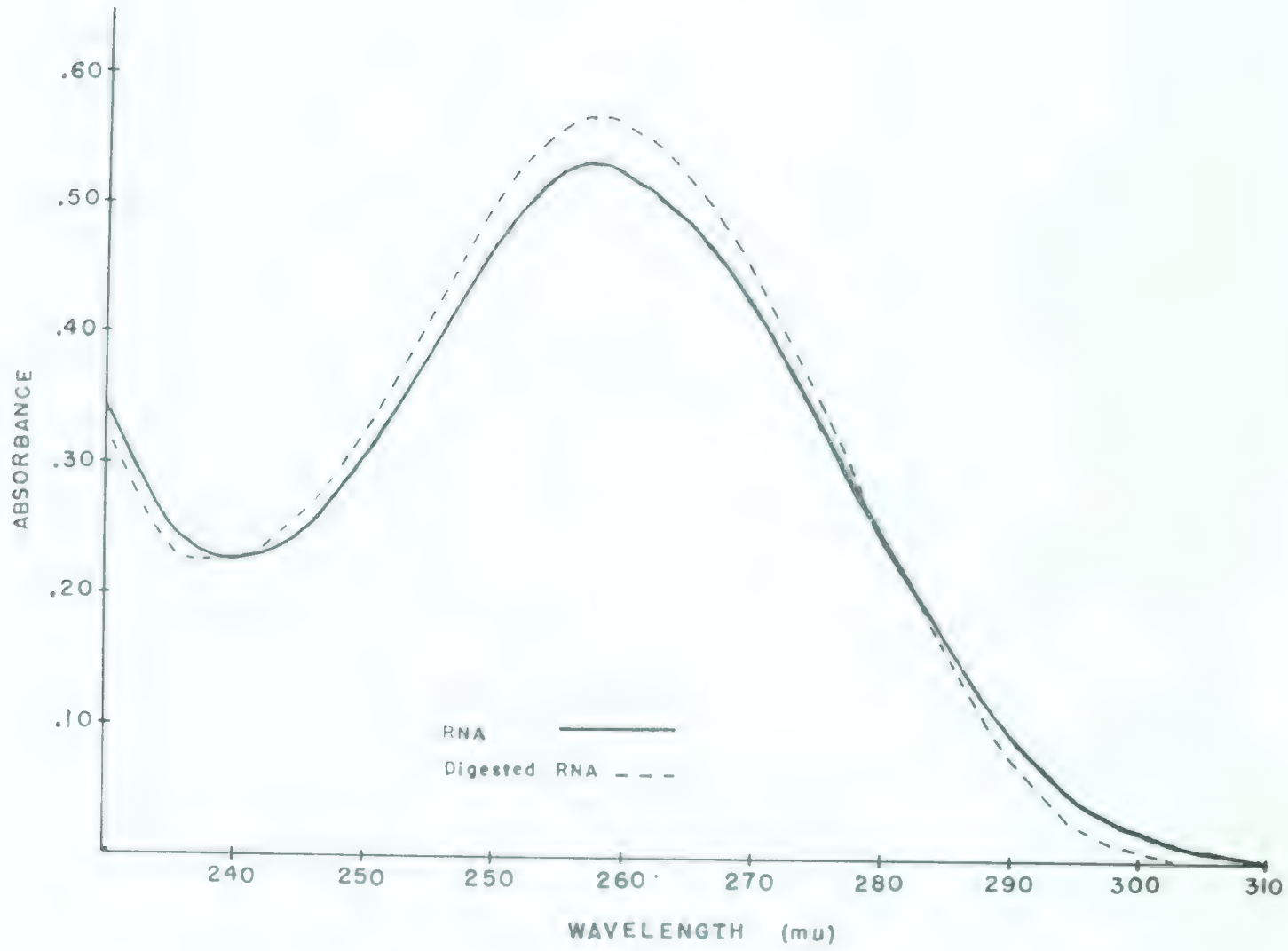


Fig. 1 A comparison of undigested and digested yeast RNA. The absorption spectrum of 20 ug/ml RNA is represented by the solid curve. The broken line curve shows the spectrum of 20 ug/ml yeast RNA digested with 0.8 ug/ml RNase.

The procedures for this method consisted of mixing the contents of two test tubes. The first contained 1 - 2 ml of 100 ug/ml soluble RNA in 0.1 M acetate buffer, pH 5, and the second, 2 ml of 0.5 ug/ml RNase in 0.1 M acetate buffer, pH 5. Where kinetin was used the 2 ml of soluble RNA was mixed with 2 ml of buffer solution containing 0.5 ug/ml RNase and 0.125 ug/ml to 4 ug/ml kinetin. Mixing was done by pouring the contents of one test tube into the other and this was repeated three times. The mixing was done gently to avoid unnecessarily harsh treatments. The mixed solution was immediately transferred into a 1 cm sq. quartz cuvette and an absorbance reading was taken at 260 mu every minute for a total time of 5 to 10 minutes, depending on the reaction rate. This method is limited to RNA concentrations not exceeding 50 ug/ml since the upper limit of spectrophotometric range is approached at that concentration.

The method of Kalnitsky, Hummel, Resnick, Carter, Barnett, and Dierks (24) was used for RNA concentrations exceeding 50 ug/ml. This method is based on the liberation of acid-soluble oligonucleotides from yeast RNA upon hydrolysis of RNase. Because this method requires precipitation of proteins by 25% perchloric acid, RNA concentrations smaller than 50 ug/ml may be drastically hydrolyzed (compared proportionately to higher RNA concentrations), thus affecting the final quantitative assay of enzyme activity.

The procedures for this method of assay again involved mixing the contents of two test tubes, one containing 2 ml of 200 - 100 ug/ml soluble yeast RNA in 0.1 M acetate buffer, pH 5, and the other containing 2 ml of 0.5 - 5 ug/ml RNase in the same buffer

solution. Where effects of kinetin were studied, 2 ml of RNA solution was mixed with a 2 ml solution containing 2 - 25 ug/ml kinetin and 0.5 - 5 ug/ml RNase. The reaction mixture was incubated at 37° C for 4 minutes. The reaction was immediately stopped by adding 1 ml of 0.75% uranium acetate in 25% $HClO_4$. The mixture was cooled at 0° C and the precipitate formed was centrifuged down at 9,000 g for 10 minutes at 4° C. An aliquot (.1 or 1 ml) of the supernatant was made up to 3 ml with distilled water and the aborbance was read at 260 mu with a Beckman DK_1 spectrophotometer using 1 cm sq. quartz cuvette.

Results

1. Preliminary Studies on RNase Activity

Preliminary studies of the effect of kinetin on RNase activity using yeast RNA as substrate gave erratic results. Although there were indications that kinetin inhibited ribonuclease activity, inconsistency in the experimental results made evaluation inconclusive. The purity of the yeast RNA (purchased from Nutritional Biochemical Corporation) was questionable. Therefore, a highly purified yeast, soluble RNA, was obtained from General Biochemicals. The results of ensuing experiments are based on the use of soluble yeast RNA (S-RNA) as substrate.

Holden and Pirie (22) found pH 5.5 to be optimum for tobacco leaf RNase activity. RNase from animal sources has been reported to possess two optimal pH's, approximately 5.5 and 8 respectively (11). Other workers (24, 29) have used pH 5 buffers

for RNase activity studies. Therefore, the pH of the reaction mixture was maintained at 5 throughout the experiments using 0.1 M acetate buffer.

The optimum temperature was found to be in the region of 25 to 37° C (Fig. 2), which is in agreement with the work of others (11, 22, 24). Since the rate of digestion was accelerated at 37° C, this temperature was used in the kinetic studies.

A suitable concentration of s-RNA was established to be in the region of 25 to 50 ug/ml where continuous spectrophotometric assay was employed. Higher concentrations of s-RNA (50 ug/ml to 10,000 ug/ml) can be used for the acid soluble oligonucleotide method of RNase assay (24).

The optimum RNase concentration using 25 to 50 ug/ml s-RNA as substrate was found to be in the region of 0.3 to 0.7 ug/ml (Fig. 3).

2. The Effect of Kinetin Concentration on RNase Activity

In order to determine the effect of kinetin on RNase activity, it was necessary to test a wide range of kinetin concentrations. A continuous assay method was used measuring the increase in absorbance at 260 mu every minute up to 8 minutes from zero time. The substrate and enzyme solutions were incubated at 37° C for 5 minutes prior to mixing. The contents of the two test tubes were thoroughly mixed, but unnecessary agitation which may cause reduction in RNase activity was avoided. A stop watch was started at the time of mixing. The reaction mixture was poured into a 1 cm sq. quartz cuvette and absorbance readings were taken every minute.

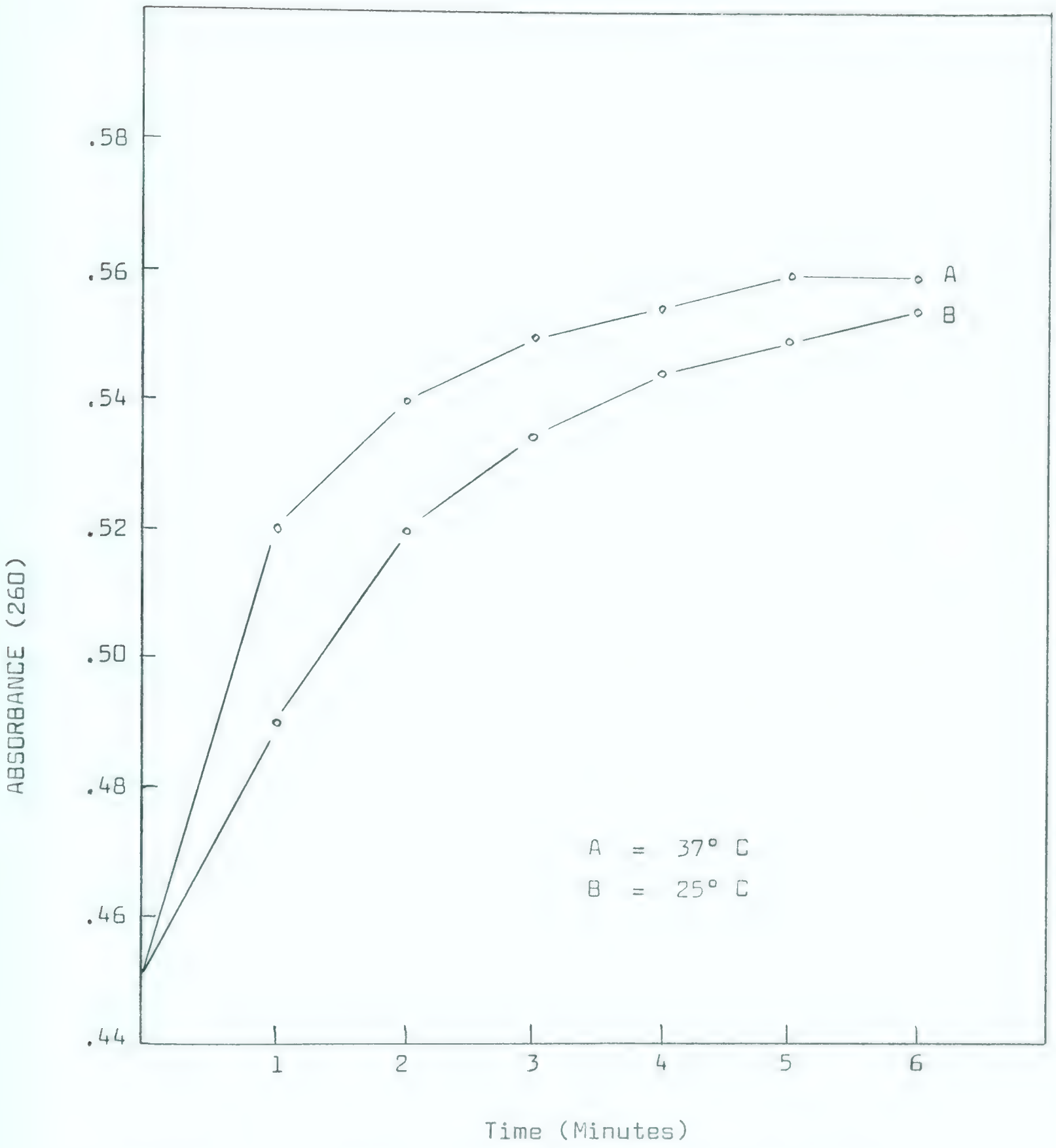


Fig. 2 The effect of temperature on RNase activity. 25 ug/ml s-RNA digested with 0.5 ug/ml RNase in 0.1 M acetate buffer, pH 5 at 25° C and 37° C.

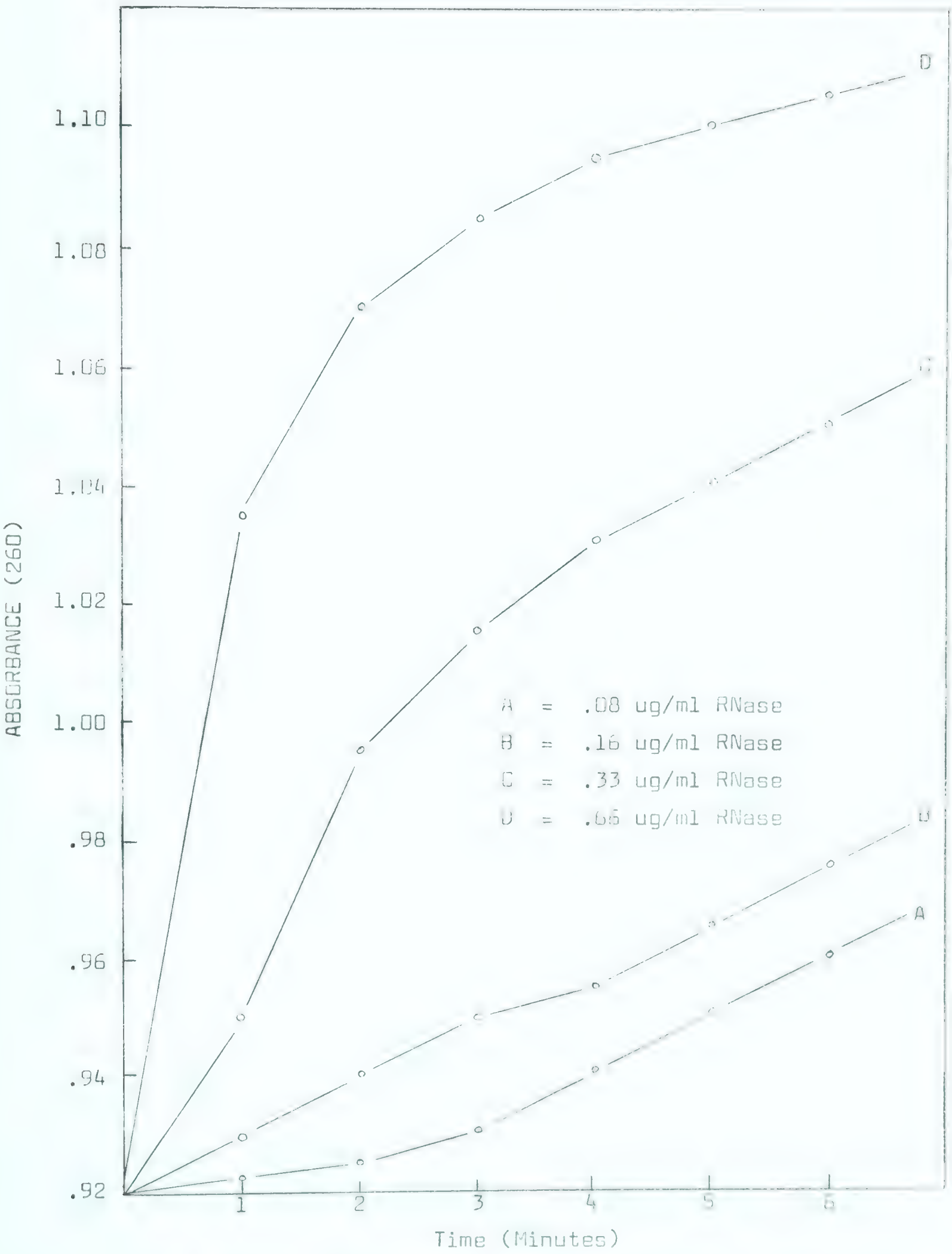


Fig. 3 RNase concentration curves. 50 ug/ml s-RNA digested with .08, .16, .33, and .66 ug/ml RNase in 0.1 M acetate buffer, pH 5 at 37° C.

Experiment 1

The results of the first trial in Experiment #1 (Table XIIIB) indicate that 5 ug/ml kinetin concentration or 1.25 ug/ml final concentration after mixing the contents of two test tubes, inhibited RNase activity, whereas kinetin at .125, .250, .50 and 1.0 ug/ml final concentrations did not appear to influence the activity.

Reproducibility of the results in trial #1 was tested by performing another trial. In the second trial, Experiment #1 (Table XIIIB), final kinetin concentrations of 1.0 ug/ml and 1.25 ug/ml slightly retarded RNase activity. Inspection of the values in trial one and trial two showed that good reproducibility was obtained, the variation being approximately 1 - 2%. An absorbance difference greater than 0.01 units was taken as evidence of a true effect on enzyme activity.

The results of the two trials indicated that kinetin concentrations above 1.0 ug/ml were inhibitory to RNase activity. Therefore, another trial was conducted using 1.0, 1.25, 1.5, and 2.0 ug/ml concentrations of kinetin. The results are tabulated in Tables XIV A and B and plotted in graph form in Figure 4.

Since slight variations existed in each of the three previous trials, a fourth trial was conducted using 1, 1.5, and 2 ug/ml kinetin concentrations. Five runs were made and an average of these are tabulated in Tables XV A and B and plotted in Figure 5.

TABLE XIIIA

Effects of kinetin on RNase activity

Experiment #1

					Absorbance Reading at 260 mu					
Trial	Test Tube #1		Test Tube #2		Time in Minutes					
#1	RNA	Buffer	RNase	Kinetin	1	2	3	4	5	6
A	1 ml	3 ml			.460	.460	.465	.465	.465	.465
B	1 ml	2 ml			.525	.545	.555	.560	.565	.565
C	1 ml	1 ml	1 ml	1 ml (.5 ug)	.535	.555	.565	.570	.570	.575
D	1 ml	1 ml	1 ml	1 ml (1 ug)	.540	.555	.565	.565	.570	.575
E	1 ml	1 ml	1 ml	1 ml (2 ug)	.560	.580	.590	.595	.600	.600
F	1 ml	1 ml	1 ml	1 ml (4 ug)	.600	.620	.630	.640	.645	.645
G				1 ml (5 ug)	.600	.620	.630	.640	.645	.645
Trial										
#2	RNA	Buffer	RNase	Kinetin	1	2	3	4	5	6
A	1 ml	3 ml			.450	.455	.460	.460	.460	.460
B	1 ml	2 ml			.520	.540	.555	.555	.560	.560
C	1 ml	1 ml	1 ml	1 ml (.5 ug)	.535	.545	.555	.560	.565	.570
D	1 ml	1 ml	1 ml	1 ml (1 ug)	.535	.555	.565	.565	.570	.575
E	1 ml	1 ml	1 ml	1 ml (2 ug)	.560	.580	.590	.595	.595	.600
F	1 ml	1 ml	1 ml	1 ml (4 ug)	.590	.610	.620	.625	.630	.630
G	1 ml	1 ml	1 ml	1 ml (5 ug)	.605	.625	.635	.640	.645	.650

	Buffer	Kinetin	Absorance at 260 mu
1	3 ml	1 ml (.5 ug)	.010
2	3 ml	1 ml (1 ug)	.015
3	3 ml	1 ml (2 ug)	.035
4	3 ml	1 ml (4 ug)	.075
5	3 ml	1 ml (5 ug)	.095

Conditions: Temperature was maintained at 37° C. RNA, RNase, and Kinetin were dissolved in 0.1 M acetate buffer, pH 5. RNA concentration was 100 ug/ml or 25 ug/ml*. RNase concentration was 2 ug/ml or 0.5 ug/ml*. Kinetin concentrations were 0.5, 1, 2, 4, and 5 ug/ml or .125, 1.66, .50, 1, and 1.25 ug/ml*.

* Refers to final concentration per ml reaction mixture (after mixing the contents of the two test tubes).

TABLE XIIIB

Effects of kinetin on RNase activity

Kinetin absorbance values subtracted from values in Table XIIIA

Experiment #1

Trial	Test Tube #1		Test Tube #2		Absorbance Reading at 260 mu Time in Minutes					
#1	RNA	Buffer	RNase	Kinetin	1	2	3	4	5	6
A	1 ml	3 ml			.460	.460	.465	.465	.465	.465
B	1 ml	2 ml			.525	.545	.555	.560	.565	.565
C	1 ml	1 ml	1 ml	1 ml (.5 ug)	.525	.545	.555	.560	.560	.565
D	1 ml	1 ml	1 ml	1 ml (1 ug)	.525	.540	.550	.550	.555	.560
E	1 ml	1 ml	1 ml	1 ml (2 ug)	.525	.545	.555	.560	.565	.565
F	1 ml	1 ml	1 ml	1 ml (4 ug)	.525	.545	.555	.565	.565	.565
G	1 ml	1 ml	1 ml	1 ml (5 ug)	.505	.535	.545	.545	.550	.550
Trial #2	RNA	Buffer	RNase	Kinetin	1	2	3	4	5	6
A	1 ml	3 ml			.450	.455	.460	.460	.460	.460
B	1 ml	2 ml			.520	.540	.550	.555	.555	.560
C	1 ml	1 ml	1 ml	1 ml (.5 ug)	.525	.535	.545	.550	.555	.560
D	1 ml	1 ml	1 ml	1 ml (1 ug)	.520	.540	.550	.555	.560	.560
E	1 ml	1 ml	1 ml	1 ml (2 ug)	.525	.545	.555	.560	.560	.565
F	1 ml	1 ml	1 ml	1 ml (4 ug)	.515	.535	.545	.550	.555	.555
G	1 ml	1 ml	1 ml	1 ml (5 ug)	.515	.530	.540	.545	.550	.555

Final Concentrations: RNA - 25 ug/ml

RNase - 0.5 ug/ml

Kinetin - .125, .25, .50, 1.0 and 1.25 ug/ml.

TABLE XIVA

Effects of kinetin on RNase activity

Experiment #1

Trial 3

Trial	Test Tube #1		Test Tube #2		Absorbance Reading at 260 mμ Time in Minutes					
#1	RNA	Buffer	RNase	Kinetin	1	2	3	4	5	6
A	1 ml	3 ml			.445	.445	.445	.445	.445	.445
B	1 ml	2 ml	1 ml		.515	.535	.545	.550	.555	.555
C	1 ml	1 ml	1 ml	1 ml (4 ug)	.585	.605	.615	.620	.625	.625
D	1 ml	1 ml	1 ml	1 ml (5 ug)	.600	.620	.630	.635	.640	.640
E	1 ml	1 ml	1 ml	1 ml (6 ug)	.615	.635	.645	.650	.655	.655
F	1 ml	1 ml	1 ml	1 ml (8 ug)	.640	.660	.670	.675	.680	.680

	Buffer	Kinetin	Absorbance Values
1	3 ml	1 ml (4 ug)	.075
2	3 ml	1 ml (5 ug)	.095
3	3 ml	1 ml (6 ug)	.115
4	3 ml	1 ml (8 ug)	.150

TABLE XIVB

					Absorbance After Kinetin Values Subtracted Time in Minutes					
	RNA	Buffer	RNase	Kinetin	1	2	3	4	5	6
A	1 ml	3 ml			.445	.445	.445	.445	.445	.445
B	1 ml	2 ml	1 ml		.515	.535	.545	.550	.555	.555
C	1 ml	1 ml	1 ml	1 ml (4 ug)	.510	.530	.540	.545	.550	.550
D	1 ml	1 ml	1 ml	1 ml (5 ug)	.505	.525	.535	.540	.545	.545
E	1 ml	1 ml	1 ml	1 ml (6 ug)	.500	.520	.530	.535	.540	.540
F	1 ml	1 ml	1 ml	1 ml (8 ug)	.490	.510	.520	.525	.530	.530

Conditions: Temperature was maintained at 37° C. All reagents dissolved in 0.1 M acetate buffer, pH 5. RNA concentration was 100 ug/ml or 25 ug/ml*. RNase concentration was 2 ug/ml or 0.5 ug/ml*. Kinetin concentrations were 4, 5, 6, and 8 ug/ml or 1, 1.25, 1.5 and 2 ug/ml*.

* Refers to final concentration per ml reaction mixture.

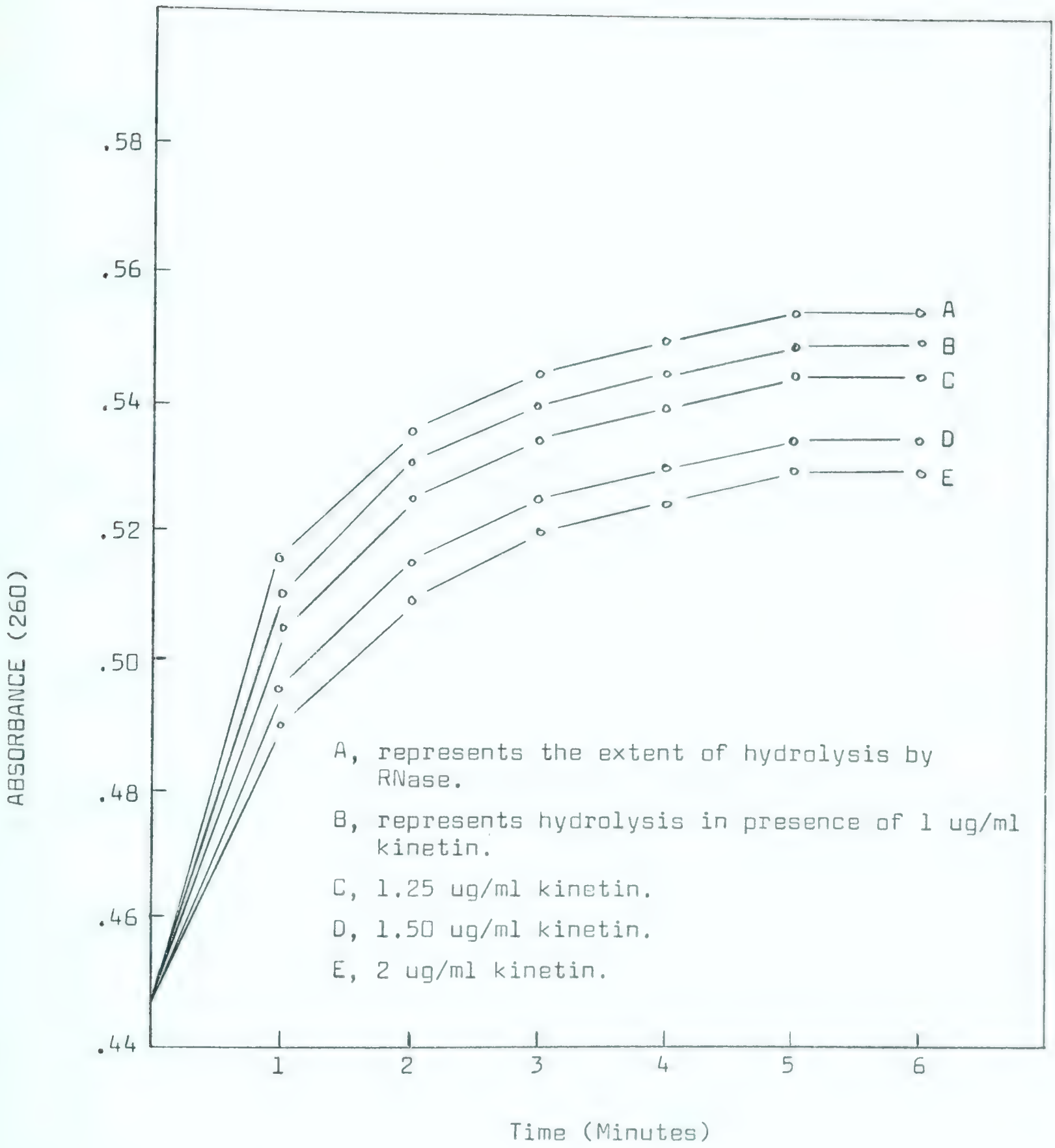


Fig. 4 Inhibiting effects of kinetin on s-RNA degradation with RNase. 25 ug/ml s-RNA digested with 0.5 ug/ml RNase in the presence of 0, 1.0, 1.25, 1.50, and 2.0 ug/ml kinetin in 0.1 M acetate buffer, pH 5 at 27° C.

TABLE XVA

Effect of kinetin on RNase activity

Experiment #1

Trial #4 (Average of five runs)

	Test Tube #1		Test Tube #2		Absorbance Reading at 260 mu Time in Minutes					
	RNA	Buffer	RNase	Kinetin	1	2	3	4	5	6
A	1 ml	3 ml			.445	.445	.445	.450	.450	.450
B	1 ml	2 ml	1 ml		.520	.540	.550	.555	.560	.570
C	1 ml	1 ml	1 ml	1 ml (4 ug)	.580	.600	.610	.620	.625	.630
D	1 ml	1 ml	1 ml	1 ml (6 ug)	.615	.635	.645	.650	.655	.660
E	1 ml	1 ml	1 ml	1 ml (8 ug)	.635	.660	.670	.680	.685	.690

	Buffer	Kinetin	Absorance Values at 260 mu
1	3 ml	1 ml (4 ug)	.075
2	3 ml	1 ml (6 ug)	.115
3	3 ml	1 ml (8 ug)	.150

TABLE XVB

	RNA	Buffer	RNase	Kinetin	Absorbance After Kinetin Values Subtracted 1	2	3	4	5	6
A	1 ml	3 ml			.445	.445	.445	.450	.450	.450
B	1 ml	2 ml	1 ml		.520	.540	.550	.550	.555	.570
C	1 ml	1 ml	1 ml	1 ml (4 ug)	.505	.525	.535	.545	.550	.555
D	1 ml	1 ml	1 ml	1 ml (6 ug)	.500	.520	.530	.535	.540	.545
E	1 ml	1 ml	1 ml	1 ml (8 ug)	.485	.510	.520	.530	.535	.540

Conditions: Temperature was maintained at 37° C. All reagents were dissolved in 0.1 M acetate buffer, pH 5. RNA concentration was 100 ug/ml or 25 ug/ml*. RNase was 2 ug/ml or 0.5 ug/ml*. Kinetin concentrations were 4, 6, and 8 ug/ml or 1, 1.5, 2 ug/ml*.

* Refers to final concentration per ml of reaction mixture.

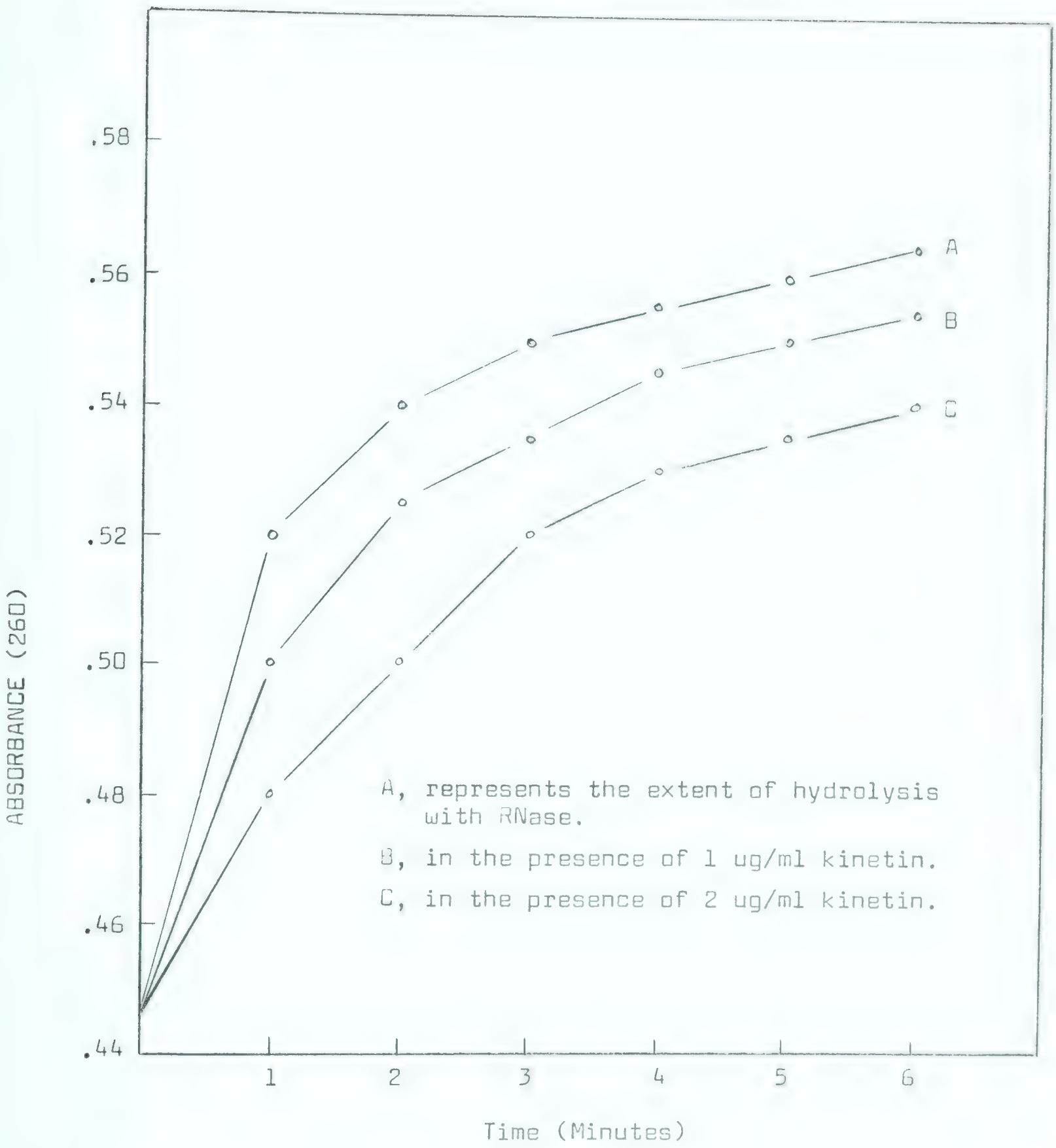


Fig. 5 Inhibiting effects of kinetin on 25 ug/ml s-RNA degradation with 0.5 ug/ml RNase in the presence of 0, 1, and 2 ug/ml kinetin in 0.1 M acetate buffer, pH 5 at 37° C.

The average of the five runs gave representative values and the inhibitory effects of kinetin were differentiated as indicated by the graph in Figure 5. If the absorbance value for RNA (A) is subtracted from B, C, D, and E, this will give an indication of the amount of nucleotide liberated upon hydrolysis by RNase in the presence of kinetin. For example, if the inhibition of RNase activity is compared after 4 minutes digestion, the following is noted:

		Absorbance
Digestion by RNase	B - A = .555 - .450	= .105
Digestion in presence 1 ug/ml	C - A = .545 - .450	= .095
Digestion in presence 1.5 ug/ml	D - A = .535 - .450	= .085
Digestion in presence 2.0 ug/ml K	E - A = .530 - .450	= .080

The RNase activities in the presence of 1, 1.5, and 2.0 ug/ml kinetin were 90%, 81%, and 76%, respectively. Thus, inhibitions as the result of kinetin concentrations were 10%, 19% and 24%.

Experiment 2

This experiment was conducted to study the effects of higher concentrations of s-RNA and kinetin on RNase activity. The concentration of s-RNA was doubled to 50 ug/ml as compared to the 25 ug/ml used in Experiment 1. Kinetin concentrations of 2 and 4 ug/ml were used and the RNase concentration was retained at 0.5 ug/ml. Furthermore, the validity of the method of subtracting kinetin absorbance values from the absorbance values obtained from the RNA + RNase + kinetin reaction used in Experiment 1 was tested.

The absorbance values of kinetin at 2 ug/ml and 4 ug/ml in buffer were .145 and .290 respectively (Table XVIA). The following shows that kinetin added to RNA did not appear to cause interaction.

Absorbance value for RNA = .915
Absorbance value for RNA + 2 ug/ml kinetin = 1.065
Absorbance value for RNA + 4 ug/ml kinetin = 1.220
Absorbance value of 2 ug/ml K = 1.065 - .915 = .150
Absorbance value of 4 ug/ml K = 1.220 - .915 = .305

The above values for kinetin absorbance are only slightly higher than those obtained where kinetin in buffer is measured. It therefore, appears reasonably valid to subtract the kinetin absorbance values directly from the absorbance values obtained where kinetin was present in the enzyme-substrate reactions.

The results of the two trials in Experiment 2 indicate that there is very little difference in the inhibition of RNase with kinetin concentrations of 2 and 4 ug/ml. Trial 1 and 2 again show good reproducibility in the results. The results of trial 1 are shown in graph form in Figure 6. In trial 1, after a digestion period of 4 minutes, the inhibitions as a result of 2 and 4 ug/ml kinetin are as follows.

B - A = .165 = increase in absorption by RNase digestion.
C - A = .140 (presence of 2 ug/ml kinetin) = 85%
D - A = .45 (presence of 4 ug/ml kinetin) = 88%

The inhibition as a result of kinetin is 15% at 2 ug/ml and 12% at 4 ug/ml.

Experiment 3

In this experiment the results of Experiment 2 were checked by using the acid-soluble oligonucleotide method. The concentrations of s-RNA and RNase were the same but the concentration of kinetin was limited to 2 ug/ml only. The results are tabulated in Tables XVII A and B.

TABLE XVIA

Effects of kinetin on RNase activity

Experiment #2

Trial	Test Tube #1		Test Tube #2		Absorbance Readings at 260 mu Time in Minutes					
#1	RNA	Buffer	RNase	Kinetin	1	2	3	4	5	6
A	2 ml	2 ml			.915	.915	.915	.915	.915	.920
B	2 ml	1 ml	1 ml		1.010	1.050	1.070	1.080	1.090	1.100
C	2 ml		1 ml	1 ml (8 ug)	1.125	1.165	1.185	1.200	1.215	1.220
D	2 ml		1 ml	1 ml (16 ug)	1.265	1.305	1.335	1.350	1.360	1.370
E	2 ml	1 ml		1 ml (8 ug)	1.065	-	-	-	-	-
F	2 ml	1 ml		1 ml (16 ug)	1.215	-	-	-	-	-
Trial #2	RNA	Buffer	RNase	Kinetin	1	2	3	4	5	6
A	2 ml	2 ml			.915	.915	.915	.915	.920	.920
B	2 ml	1 ml	1 ml		1.010	1.050	1.080	1.095	1.105	1.115
C	2 ml		1 ml	1 ml (8 ug)	1.115	1.155	1.180	1.200	1.210	1.220
D	2 ml		1 ml	1 ml (16 ug)	1.265	1.305	1.335	1.355	1.365	1.375
E	2 ml	1 ml		1 ml (8 ug)	1.060	1.065	1.065	-	-	-
F	2 ml	1 ml		1 ml (16 ug)	1.215	1.215	1.220	-	-	-

	Buffer	Kinetin	Absorbance Values at 260 mu
1	3 ml	1 ml (8 ug)	.145
2	3 ml	1 ml (16 ug)	.290

Conditions: Temperature was maintained at 37° C. All reagents dissolved in 0.1 M acetate buffer, pH 5. RNA concentration was 100 ug/ml or 50 ug/ml*. RNase concentration was 2 ug/ml or 0.5 ug/ml*. Kinetin concentrations were 8 and 16 ug/ml or 2 and 4 ug/ml*.

* Final concentration refers to concentration of the materials per ml of of reaction mixture.

TABLE XVIB

Effects of kinetin on RNase activity

Kinetin absorbance values subtracted from values in Table XVIA

Experiment #2

					Absorbance Readings at 260 mu					
Trial	Test Tube #1		Test Tube #2		Time in Minutes					
#1	RNA	Buffer	RNase	Kinetin	1	2	3	4	5	6
A	2 ml	2 ml			.915	.915	.915	.915	.915	.920
B	2 ml	1 ml	1 ml		1.010	1.050	1.070	1.080	1.090	1.100
C	2 ml		1 ml	1 ml (8 ug)	.980	1.020	1.040	1.055	1.070	1.075
D	2 ml		1 ml	1 ml (16 ug)	.975	1.015	1.045	1.060	1.070	1.080
E	2 ml	1 ml		1 ml (8 ug)	.920	-	-	-	-	-
F	2 ml	1 ml		1 ml (16 ug)	.925	-	-	-	-	-
Trial										
#2	RNA	Buffer	RNase	Kinetin	1	2	3	4	5	6
A	2 ml	2 ml			.915	.915	.915	.920	.920	.920
B	2 ml	1 ml	1 ml		1.010	1.050	1.080	1.095	1.105	1.115
C	2 ml			1 ml (8 ug)	.970	1.010	1.035	1.055	1.065	1.075
D	2 ml			1 ml (16 ug)	.975	1.015	1.045	1.060	1.070	1.080
E	2 ml	1 ml		1 ml (8 ug)	.920	-	-	-	-	-
F	2 ml	1 ml		1 ml (16 ug)	.925	-	-	-	-	-

Final Concentrations: RNA - 50 ug/ml
RNase - 0.5 ug/ml
Kinetin - 2 and 4 ug/ml.

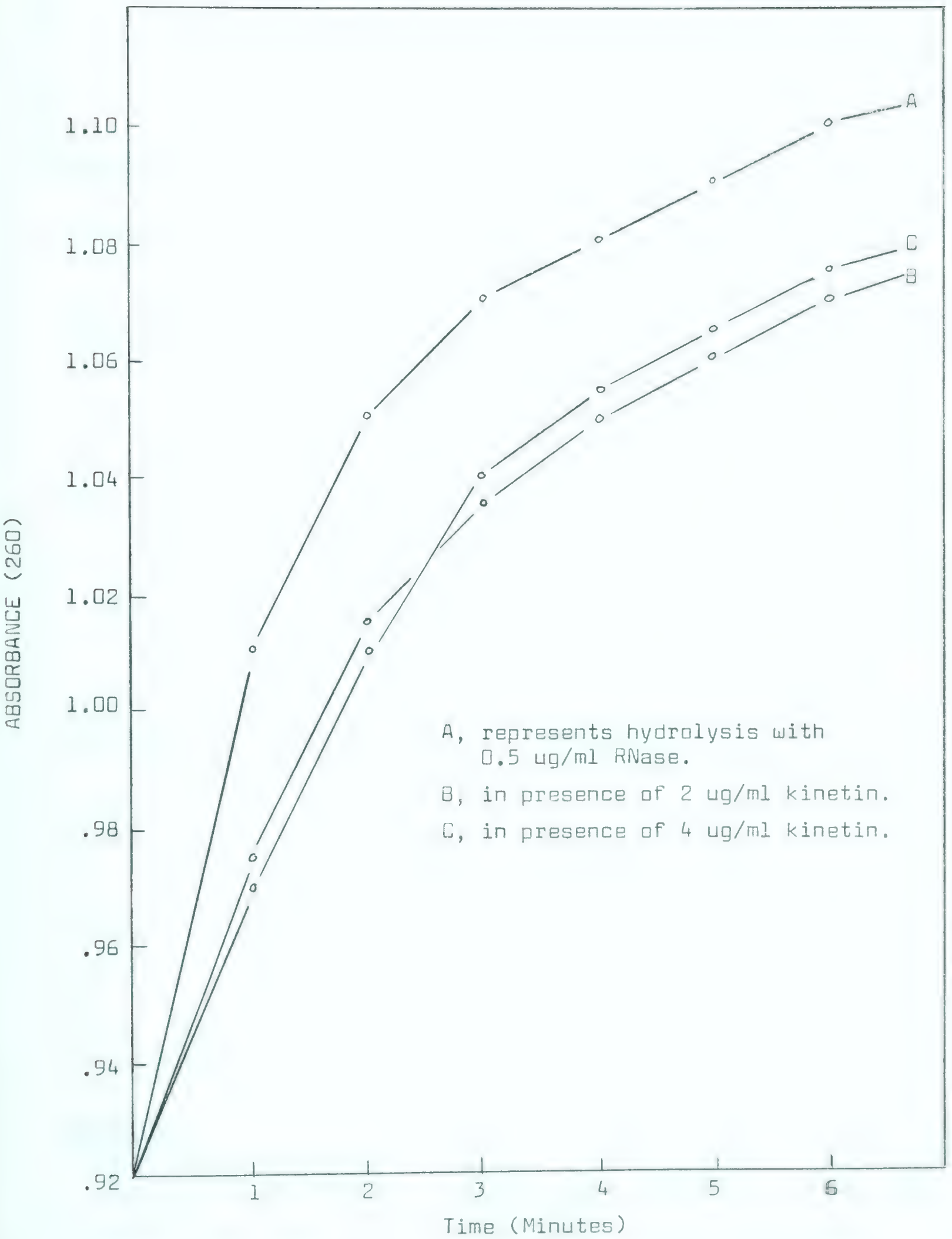


Fig. 6 Inhibiting effects of kinetin on s-RNA degradation with RNase. 50 ug/ml s-RNA digested with 0.5 ug/ml RNase in the presence of 0, 2, and 4 ug/ml kinetin in 0.1 M acetate buffer, pH 5 at 37° C.

TABLE XVIIA

Effects of kinetin on RNase activity

Oligonucleotide assay method

Experiment #3

	Test Tube #1		Test Tube #2		Absorbance Readings at 260 mu			
	RNA	Buffer	RNase	Kinetin	Trial 1	Trial 2	Trial 3	Trial 4
A	2 ml	2 ml			.390	.390	.415	.405
B	2 ml	1 ml		1 ml	.440	.445	.450	.450
C	2 ml	1 ml	1 ml		.650	.630	.640	.630
D	2 ml		1 ml	1 ml	.670	.655	.655	.660
E		4 ml			.360	.350	.355	.350
F		3 ml		1 ml	.410	.400	.390	.395

TABLE XVIIB

Absorbance after Uranium - $HClO_4$ value subtracted

	Trial 1	Trial 2	Trial 3	Trial 4	Average
A	.030	.040	.060	.055	.046
B	.080	.095	.095	.100	.093
C	.290	.280	.295	.280	.286
D	.310	.305	.310	.310	.309
E	0	0	0	0	0
F	.050	.050	.045	.045	.048

Conditions: Temperature was maintained at 37° C. Reaction mixture was incubated for 4 minutes. Reaction was stopped with 1 ml 0.75% uranium acetate in 25% $HClO_4$. All reagents were dissolved in 0.1 M acetate buffer, pH 5. RNA concentration was 100 ug/ml or 50 ug/ml*. RNase concentration was 2 ug/ml or 0.5 ug/ml*. Kinetin concentration was 8 ug/ml or 2 ug/ml*. One ml aliquot supernatant was diluted with 2 ml distilled water.

* Refers to final concentration per ml reaction mixture.

The absorbance as a result of the addition of 1 ml uranium acetate-perchloric acid is approximately 0.355. Subtracting the respective absorbance values for uranium acetate - $HClO_4$ from each trial gave actual absorbance values due to release of acid soluble materials from RNA, digested RNA, and digested RNA in presence of 2 ug/ml kinetin. The corrected values are tabulated in Table XVIIB.

The average absorbance value for kinetin in buffer is .048, addition of kinetin to RNA did not appear to cause interaction, B - A = .093 - .046 = .047. The release of oligonucleotide by RNase is indicated by the value, C - A = .286 - .046 = .240 absorbance units. The oligonucleotide liberated in presence of 2 ug/ml kinetin is quantitatively represented by the absorbance value of D - B = .309 - .093 = 0.214. This is approximately 11% reduction as the result of kinetin addition, indicating inhibition of RNase degradation of s-RNA. The inhibition obtained by 2 ug/ml kinetin after 4 minutes using the continuous assay method in Experiment 2 was 15%.

Experiment 4

The endogenous RNA extracted from approximately 250 mg of Xanthium leaf discs was reported by Osborne (47) to be in the region of 250 ug. In the studies on the effects of N^6-benzyladenine on cabbages, we found that alkali-extractable RNA from 100 mg of cabbage leaf powder exceeded 1000 ug, using soluble yeast RNA as a standard. Therefore, in order to adjust *in vitro* concentrations in relationship to naturally occurring amounts, a 10-fold increase in the RNA and RNase, and a 12.5-fold in the kinetin, over the concentration used in Experiment 3, was made.

The acid-soluble oligonucleotide method of assay for RNase activity was used as previously. The procedure was the same except that 0.1 ml of supernatant was diluted with 3 ml of distilled water rather than 1 ml made up to 3 ml in Experiment 3.

The results are tabulated in Table XVIII. The absorption due to kinetin was approximately .055 as indicated by B - A and F - E. RNase did not influence the absorbance values significantly (approximately 2.6% increase over buffer blank). The increase in absorbance value upon liberation of oligonucleotide by RNase is represented by C - A, and in the presence of kinetin, by D - B. The increase in absorbance caused by the liberation of acid-soluble oligonucleotide by RNase was .118, whereas in the presence of 25 ug/ml kinetin it was .087. This represented 74% activity or 26% inhibition.

Discussion

The results of the *in vitro* studies of the effect of kinetin on RNase activity indicate that kinetin has an inhibitory effect on the RNase degradation of soluble yeast RNA. Evidence for the inhibitory effect was obtained by the results from two methods of enzyme assay. The continuous method of assay indicated a 15% reduction when 50 ug/ml s-RNA was digested with 0.5 ug/ml RNase in the presence of 2 ug/ml kinetin. The second method of measuring the amount of acid-soluble oligonucleotide liberated under the same conditions indicated an 11% inhibition.

The higher percentage may be attributed to the presence of contaminants such as protein in the reaction mixture of the first method, but in the second method proteins were precipitated out with uranium acetate - $HClO_4$.

TABLE XVIII

Effects of kinetin on RNase activity

Experiment #4

	Test Tube #1		Test Tube #2		Absorbance Reading at 260 mu			
	RNA	Buffer	RNase	Kinetin	Trial 1	Trial 2	Trial 3	Average
A	2 ml	2 ml			.195	.190	.195	
B	2 ml	1 ml		1 ml	.245	.240	.240	
C	2 ml	1 ml	1 ml		.210	.310	.315	
D	2 ml		1 ml	1 ml	.330	.335	.330	
E		4 ml			.190	.190	.190	
F		3 ml		1 ml	.250	.245	.245	
G		3 ml	1 ml		.195	.195	.195	
				C - A	.115	.120	.120	.118
				D - B	.085	.095	.080	.087

Conditions: Temperature was maintained at 37° C. Reaction time was 4 minutes. All reagents were dissolved in 0.1 M acetate buffer, pH 5. RNA concentration was 1000 ug/ml or 500 ug/ml*. RNase concentration was 20 ug/ml or 5 ug/ml*. Kinetin concentration was 100 ug/ml or 25 ug/ml*. 0.1 ml aliquot diluted with 3 ml distilled water.

* Refers to final concentration per ml reaction mixture.

Blockage of the active sites or binding sites on the enzyme molecule by kinetin may occur, thus decreasing the rate of s-RNA degradation by RNase. Since the physiological concentration of kinins applied in the treatment of explants usually ranges from 5 to 50 ppm and since concentrations of kinetin found to be inhibitory in in vitro studies varied from 1 ug/ml (1 - 25 ppm, higher concentrations were not tested) it is reasonable to propose that the mechanism involved in the retardation of senescence in kinin-treated plants may be associated with RNA catabolism.

The results of in vitro studies strongly support the hypothesis that kinins inhibit the natural enzymatic degradation of RNA (possibly s-RNA by ribonuclease), thereby maintaining the metabolic level of s-RNA for protein synthesis in the chloroplast and, as a consequence, the chlorophyll level. The maintenance of metabolic level of s-RNA and the consequential maintenance of protein synthesis may account for the accelerated incorporation of labelled materials into the proteins of kinin-treated explants as reported by Osborne (47) and Sugiura, et al. (66).

A very recent report by Lovrekovich and Farkas (32) may have relevance to our suggestion that kinins may inhibit the RNase degradation of RNA. They found that chlorosis and protein breakdown induced by a bacterial toxin of Pseudomonas tobaci can be counteracted by kinetin. The structure of the bacterial toxin is similar to methionine. The toxin-induced breakdown is similar to the loss of chlorophyll and protein that occurs with senescence. It would appear that similar mechanisms may be involved. They

also reported that RNase activity in extracts from tobacco tissues treated with toxin-containing culture filtrates of Pseudomonas tobaci was markedly increased. This suggests that there may be synergistic effects of the toxin and RNase in degrading RNA. Since bacterially-induced breakdown was inhibited by kinetin, it may be reasonable to propose that RNA degradation by RNase was inhibited.

B. THE EFFECT OF N^6-BENZYLADENINE ON THE RIBONUCLEIC ACID LEVELS IN CABBAGE LEAVES

The retention of higher levels of RNA in kinetin-treated leaf discs has been reported for Xanthium and tobacco (47, 66). Since N^6-benzyladenine was also found to effectively retard senescence of cabbage, RNA extractions of cabbage leaves were made to determine the levels of endogenous RNA in treated and control cabbage heads.

Materials and Methods

Cylindrical plugs from N^6-benzyladenine-treated and control heads of Pennstate Ballhead cabbages that were stored at 40° F for a 10-week period were used for RNA extraction. The cabbages were dip-treated in an aqueous solution of 40 ppm N^6-benzyladenine containing 0.25 ml Tween-wo per gallon water as wetting agent. The experiment consisted of 4 uniform heads per treatment, replicated 3 times. A cork borer was used to randomly sample 10 plugs (approximately 1 cm in depth and 1 cm in diameter) from each of the 4 heads in the treatments.

There have been several methods of RNA extraction reported in recent years (6, 21, 43, 60). However, the method of Nieman and

Poulsen (43) was adopted for our use since they have modified and incorporated several methods specifically for leaf RNA extraction. Since the samples of leaf materials used were larger, the volumes of the reagents were increased accordingly.

The 40 plugs from each treatment were separated into individual leaf-disc sections and placed in 100 ml of boiling 95% ethanol for three minutes to extract chlorophyll. After cooling, the discs (in ethanol) were minced in a homogenizer for 3 minutes. The homogenate was centrifuged at 5,000 g for 10 minutes and the ethanol portion was made up to 100 ml for chlorophyll determination. The sediment was extracted by repeated suspension and centrifugation in different reagents as follows:

1. Once with 30 ml of 95% ethanol at room temperature.
2. Twice with 30 ml of 50% ethanol (adjusted to pH 5 with glacial acetic acid) at room temperature.
3. Twice with 30 ml chilled (4° C) 0.2 N $HClO_4$. The suspension held at 4° C for 15 minutes before centrifuging.
4. Once with 30 ml 95% ethanol at room temperature.
5. Twice with 30 ml boiling absolute ethanol-ether (3:1) for three minutes.
6. Once with 30 ml ether at room temperature.

After the final centrifugation the pellets were dried to leaf powder.

The next procedure was the digestion of leaf powder by alkali. A 100 mg sample of leaf powder was suspended with 5 ml of 0.3 N NaOH and held at 30° C for 15 hours. The alkali-digested residue of leaf powder was centrifuged at 7,000 g for 10 minutes.

The sediment was washed once with 5 ml of 0.3 N NaOH and again centrifuged. The alkali supernatant was pooled and made up to 10 ml volume. Two ml of 15% $HClO_4$ was added to acidify the supernatant to pH 1. The extract was held at 4° C for 45 minutes. The $HClO_4$ insoluble material which precipitated was centrifuged at 9,000 g for 10 minutes.

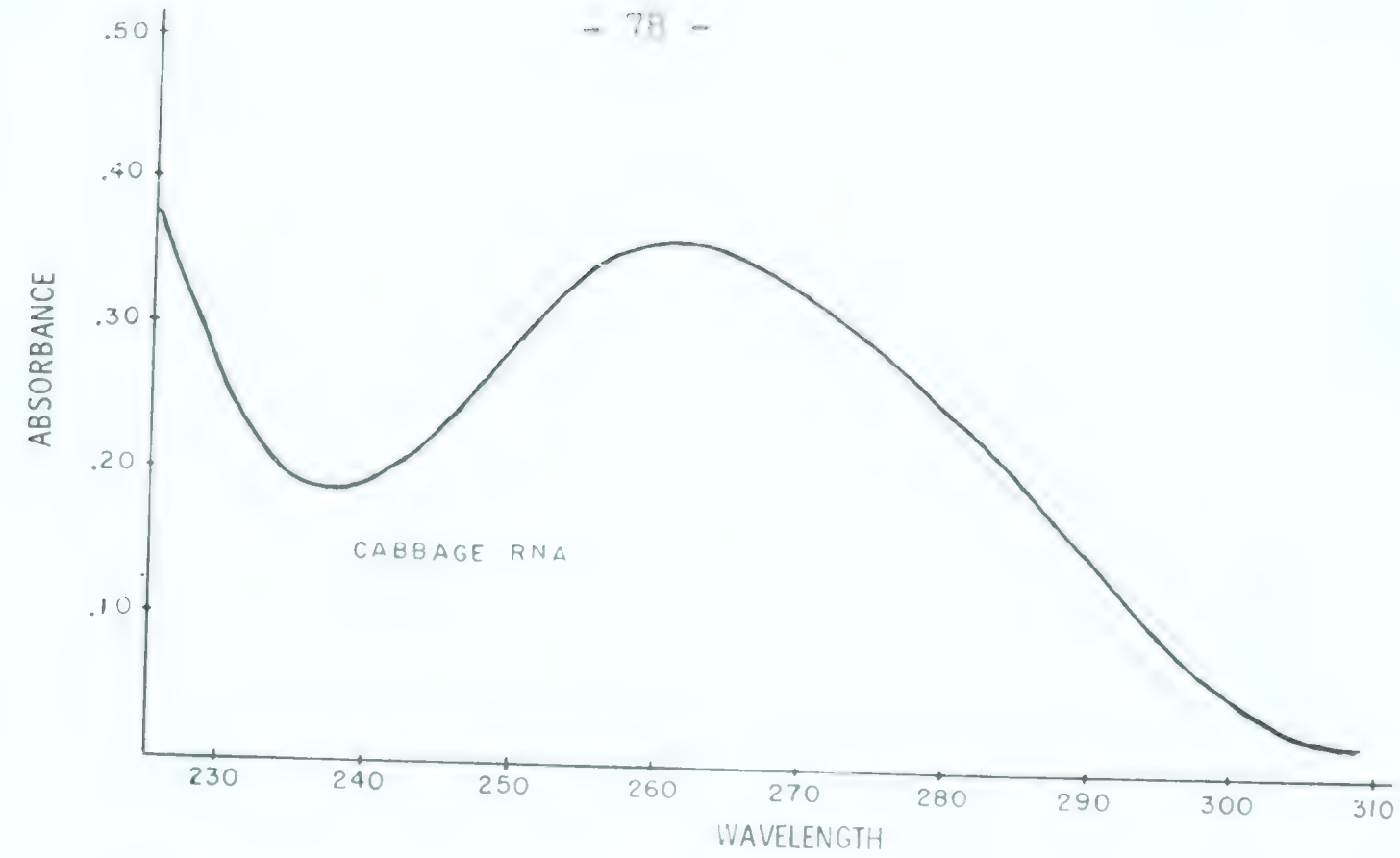
The RNA was estimated spectrophotometrically according to the method of Nieman and Poulsen (43). A ml aliquot of the supernatant was diluted with 2 ml distilled water in a 1 cm square quartz cuvette. A Beckman DK_1 recording spectrophotometer was used to measure the absorbance at 260 mu. Scanning between 310 mu to 230 mu was also done to study the characteristics of the RNA spectrum.

Results

The absorption spectra of s-RNA and extracted cabbage leaf RNA are compared in Figure 7. The spectrum of cabbage leaf RNA approaches that of radish cotyledon RNA reported by Nieman and Poulsen (43) and peanut seedling RNA reported by Cherry (6). The maximum absorption of cabbage RNA is at 260 mu and the minimum is at 240 mu, whereas the maximum and minimum for s-RNA are 260 mu and 235 mu respectively.

Levels of RNA extracted from leaves of N^6-benzyladenine-treated and control Pennstate Ballhead cabbages estimated at 260 mu are shown in Table XIXA.

The spectra of samples from three replicates are shown in Figures 8, 9, and 10. The analysis of variance (Table XIXB) indicates that the cabbages treated with N^6-benzyladenine contained significantly higher levels of RNA.



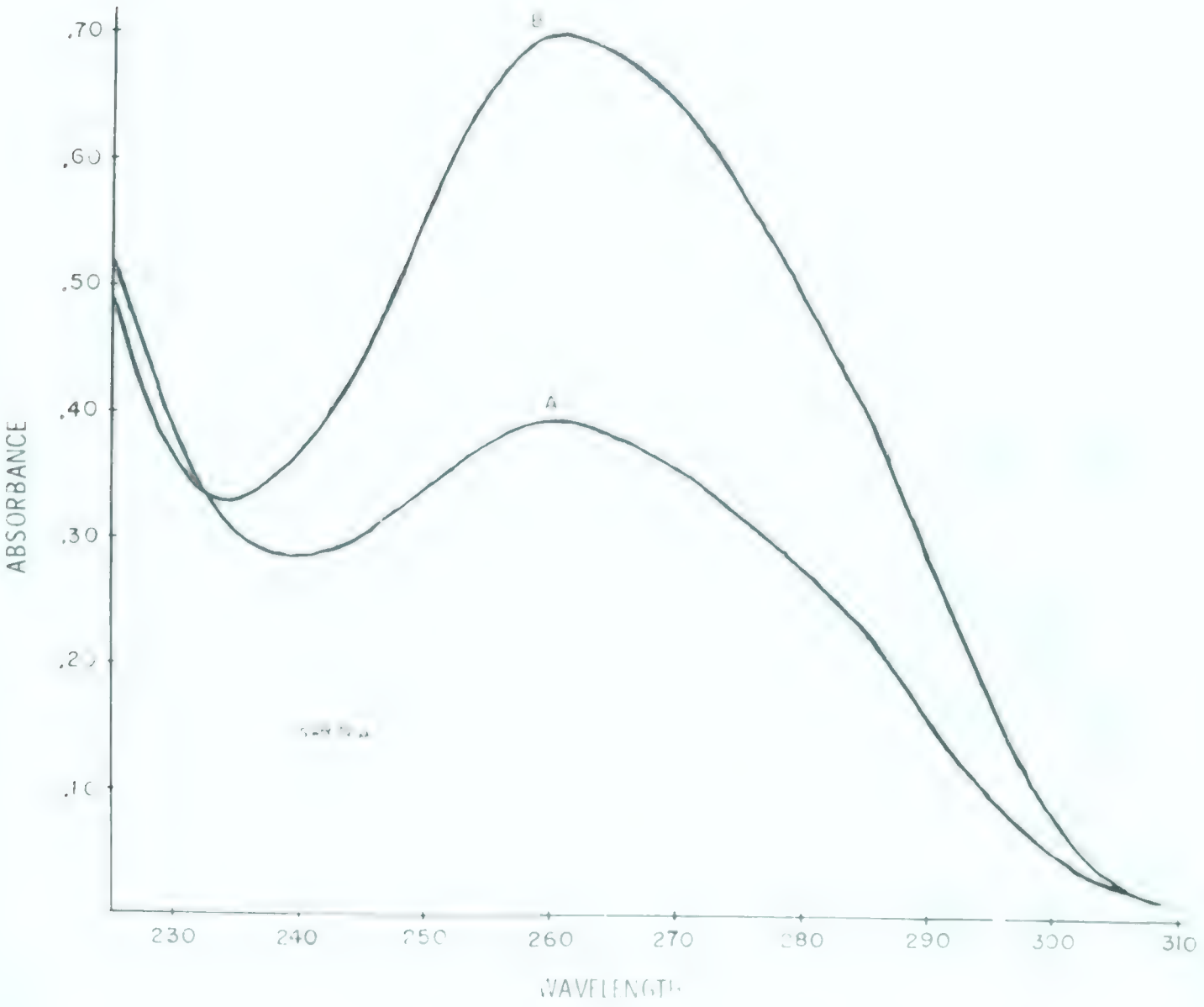


Fig. 7 A spectral comparison of cabbage leaf RNA and yeast s-RNA. A 100 mg cabbage leaf powder was digested with 0.3 NaOH at 37° C for 15 hours. 500 ug/ml and 1000 ug/ml s-RNA was treated in the same manner. A one ml aliquot was diluted with 2 ml water.

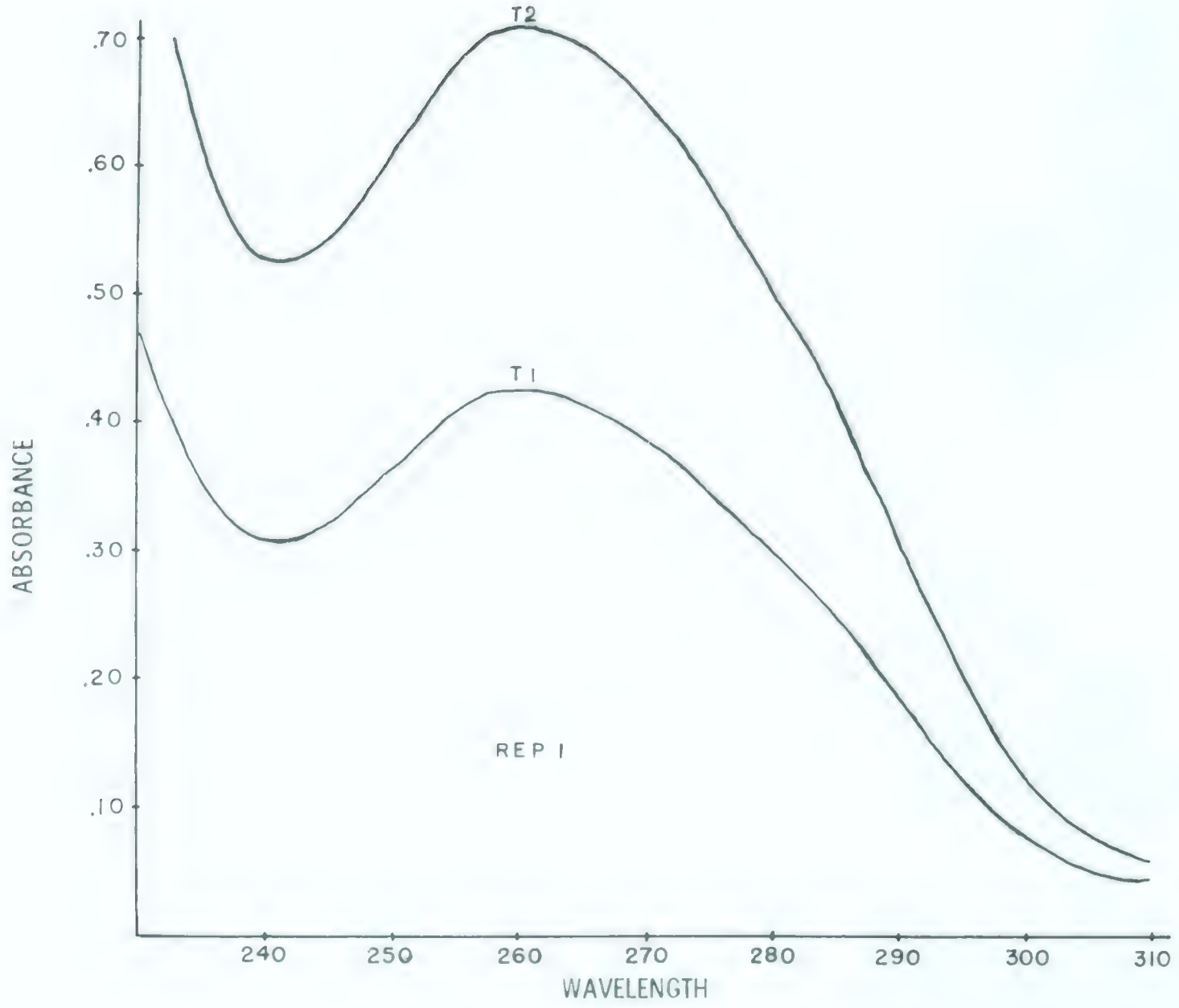


Fig. 8 Absorption spectra of cabbage leaf. RNA extracted from 100 mg leaf powder with 0.3 NaOH at 37° C for 15 hours. T_1 represents RNA extracted from control and T_2 represents RNA extracted from cabbages treated with 40 ppm N^6-benzyladenine.

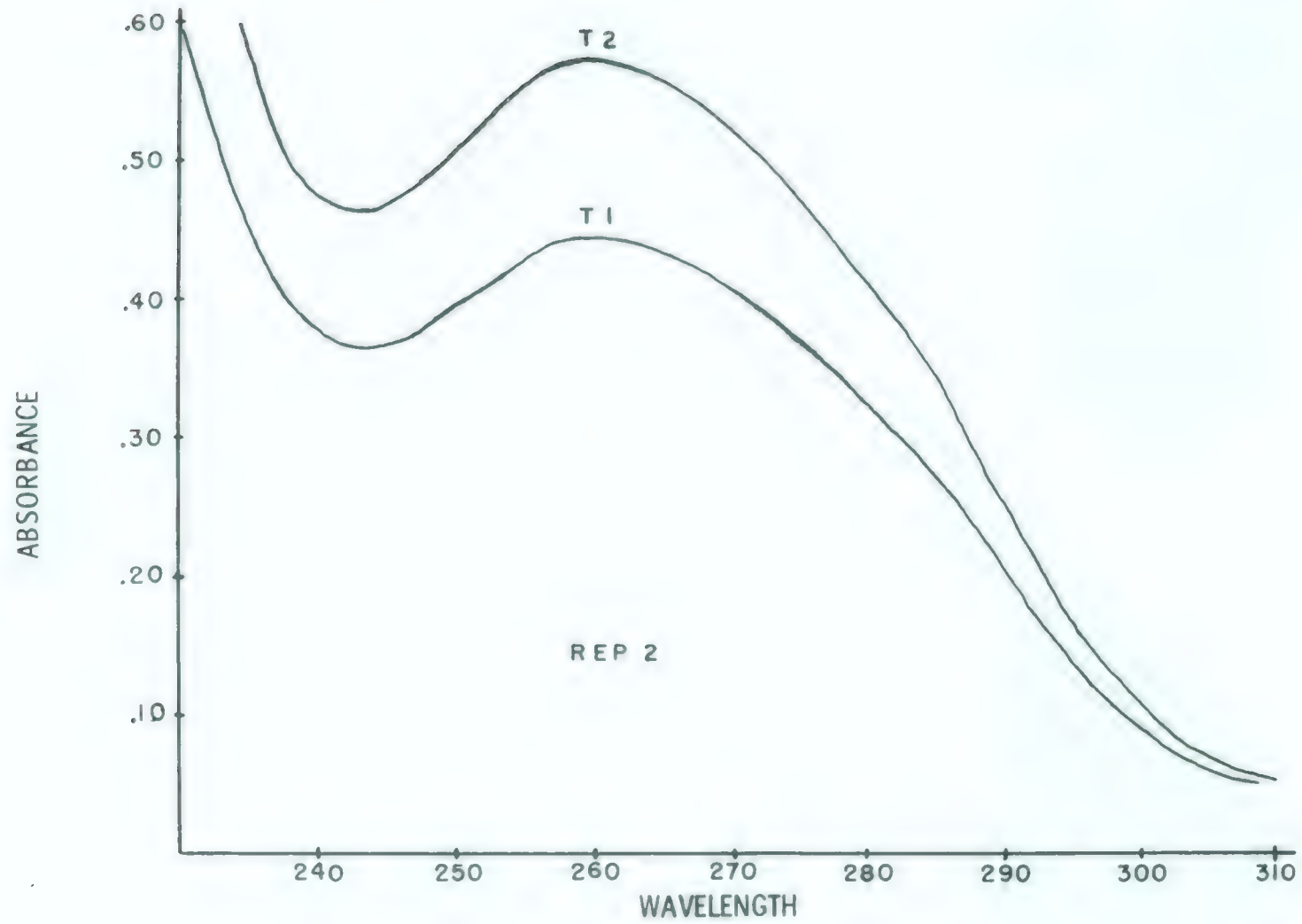


Fig. 9 Absorption spectra of cabbage leaf. RNA extracted from 100 mg leaf powder with 0.3 NaOH at 37° C for 15 hours. T_1 represents RNA extracted from control and T_2 represents RNA extracted from cabbages treated with 40 ppm N^6-benzyladenine.

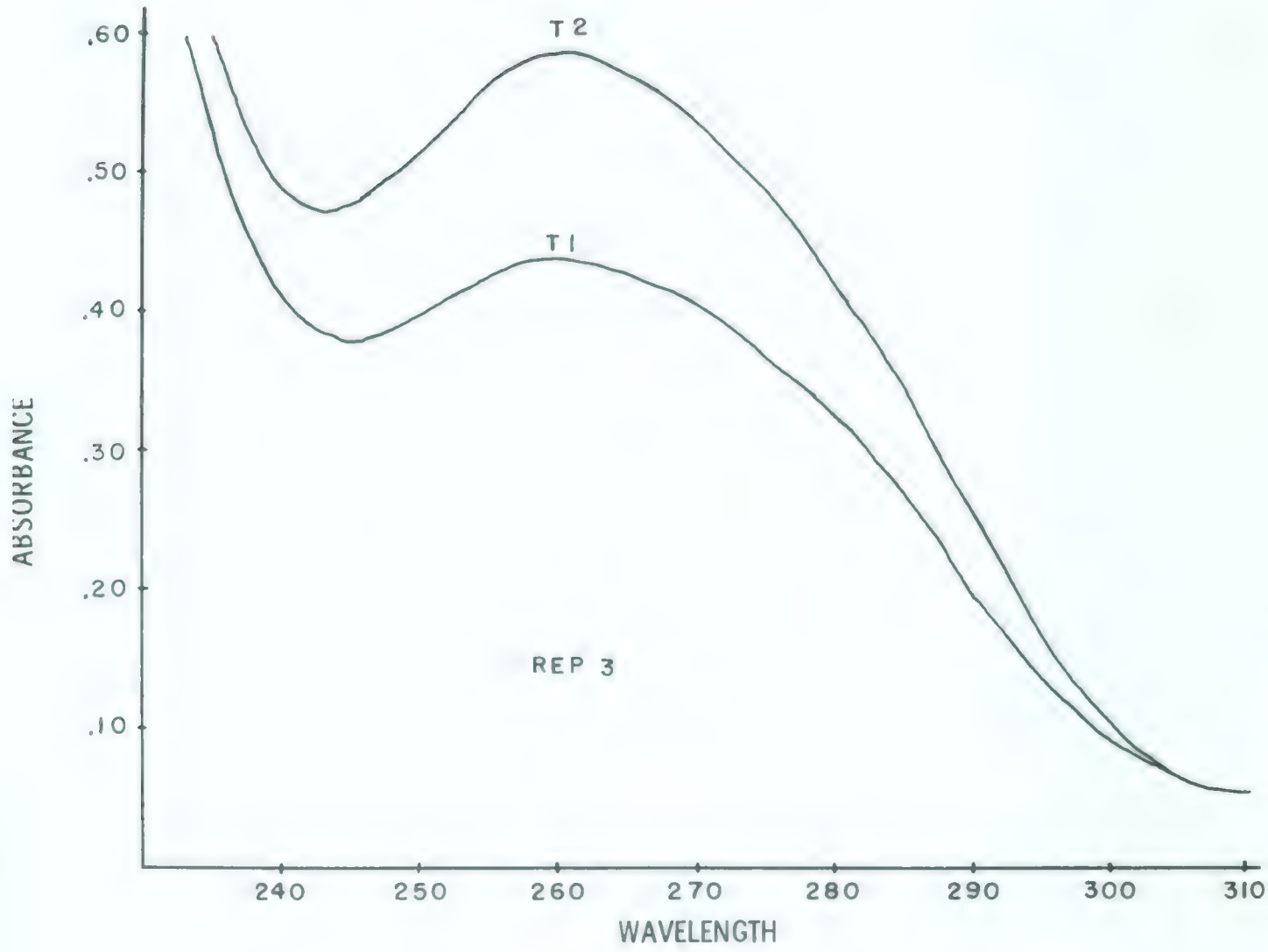


Fig. 10 Absorption spectra of cabbage leaf. RNA extracted from 100 mg leaf powder with 0.3 NaOH at 37° C for 15 hours. T_1 represents RNA extracted from control and T_2 represents RNA extracted from cabbages treated with 40 ppm N^6benzyladenine.

TABLE XIX

A. The comparison of RNA levels in N^6-benzyladenine-treated and non-treated Pennstate cabbage. RNA estimated at 260 mu. (Values are averages of two samples.)

	Replication 1	Replication 2	Replication 3
Control	.498	.443	.445
40 ppm N^6BA	.745	.580	.573

B. Analysis of variance of cabbage RNA estimated by maximum absorbance at 260 mu

Source of variation	d.f.	SS	MS	F
Replications	2	.01651		
Treatments	1	.04369	.04369	25.7*
Error	2	.00339	.00169	

F.05 (1, 2) = 18.51

* Exceeds the .05 level of significance.

TABLE XX

Comparison of RNA and chlorophyll levels in N^6-benzyladenine-treated and non-treated Pennstate cabbage

		RNA (260 mu)	Chlorophyll (663 mu)
Replication 1	Control	.425	.285
	40 ppm N^6BA	.650	.515
Replication 2	Control	.445	.220
	40 ppm N^6BA	.570	.520
Replication 3	Control	.435	.200
	40 ppm N^6BA	.575	.435

A comparison of RNA and chlorophyll extracted from N^6-benzyladenine-treated and non-treated Pennstate Wellhead cabbages is shown in Table XX. The chlorophyll and RNA levels in kinin-treated and non-treated cabbages are correlated with the degree of yellowing, indicating that, in treated cabbages, both chlorophyll and RNA are retained at a higher level.

In order to determine the precision of the sampling technique, duplicate samples were taken from each treatment (4 cabbages) and RNA estimations were done for each sample. Absorbance readings at 260 mu were made to estimate the levels of RNA in each sample within each treatment and replication.

The analysis of variance (Table XXIB) indicates that sampling error is small and statistically insignificant. The method used here of partitioning the error due to sampling gives greater precision than that of finding sample averages (Table XIX). The effect of treatment was highly significant, and it can be concluded that the RNA levels in N^6-benzyladenine-treated Pennstate cabbages were significantly higher than those of control lots.

Using the s-RNA concentrations as standards, it was estimated that kinin-treated cabbages contained approximately 750 to 1000 ug RNA per 100 mg leaf powder whereas the control contained 500 - 600 ug RNA per 100 mg leaf powder.

Discussion

The ultra-violet spectrum of RNA extracted from cabbage leaf was found to be similar to those reported for radish cotyledon and peanut seedling (6, 43). It was found that the leaf RNA

TABLE XXI

A. RNA levels of duplicate samples from N^6-benzyladenine-treated and non-treated Pennstate cabbage. Estimated at 260 mu

		Samples	
	Treatment	1	2
Replication 1	Control	.438	.525
	40 ppm N^6BA	.713	.805
Replication 2	Control	.448	.455
	40 ppm N^6BA	.645	.578
Replication 3	Control	.460	.448
	40 ppm N^6BA	.618	.598

B. Analysis of variance of RNA levels in N^6-benzyladenine-treated and non-treated Pennstate cabbages

Source of variation	d.f.	SS	MS	F
Replications	2	.0211	.0105	
Treatments	1	.1166	.1166	38.88*
Samples	1	.0006	.0006	0.20
Error	7	.0200	.0031	

F.01 (1, 7) = 12.25

* Exceeds the .01 level of significance.

extraction and estimation method of Nieman and Poulsen (43) was reasonably adequate for our use in the comparison of extractable leaf RNA in kinin-treated and control cabbages. Aproximate estimation was possible using soluble yeast RNA as standard, but slight differences in the absorption characteristics of s-RNA and cabbage leaf RNA made precise quantitative estimation of the concentrations difficult.

Maximum absorption at 260 mu was used as the basis for RNA comparison. It was found that the levels of RNA in N^6-benzyladenine treated Pennstate cabbages were significantly higher than those extracted from controls. The levels of chlorophyll correlated with the levels of RNA; the kinin-treated cabbages had higher levels of chlorophyll and RNA as compared to those of controls.

The method of sampling used was found to be acceptable and without large sampling error.

The results of the experiments indicate that RNA is maintained at a higher level in kinin-treated cabbages than in control cabbages. These results are in agreement with those of others working with Xanthium and tobacco explants (47, 66) and support the hypothesis that kinins are implicated in nucleic acid metabolism.

SUMMARY AND CONCLUSIONS

Foliar spray application of N^6-benzyladenine at the first true-leaf stage of growth brought about a retardation in the growth of the radish plants. At a concentration of 200 ppm, both leaf and hypocotyl growth was severely inhibited, causing atypical leaf development and failure of hypocotyl expansion. Lower concentrations of 20, 40, 80 and 160 ppm N^6-benzyladenine retarded the growth and development of the hypocotyl. No differences were noted in leaf and flower development.

N^6-benzyladenine was found to be effective in prolonging storage and retarding yellowing of bunched radish leaves. Dip-treated radishes (20 and 40 ppm) stored at 36° ± 2° F were still green after 55 days and control bunches showed yellowing during the same period. Of the radishes treated similarly and stored for 25 days, those treated with 40 ppm remained green and fresh, whereas the 20 ppm treatment showed slight yellowing of the leaves. The controls turned completely yellow.

N^6-benzyladenine did not appear to reduce respiration of the radish roots. There were no significant differences in weight loss of radish roots treated with 0, 50, and 100 ppm N^6-benzyladenine and stored at 36° ± 2° F for 10 weeks. No differences were noted on radishes treated similarly and stored at 46° ± 2° F.

N^6-benzyladenine effectively retarded yellowing and prolonged the storage life of several varieties of cabbage. Significant differences were found in the chlorophyll levels of Early Marvel cabbage treated with 0, 10, 20 and 30 ppm N^6-benzyladenine.

Cabbages treated with 10 and 20 ppm contained twice the chlorophyll content of the control cabbages, whereas those treated with 30 ppm contained 4 times the chlorophyll content of the control plants. Marketability of the Early Marvel cabbage was extended approximately 2 weeks by kinin application.

Cabbages (Pennstate Ballhead variety) treated with 50 ppm N^6-benzyladenine 3 weeks after harvest retained the chlorophyll content at a higher level than non-treated controls. This suggested the practical use of kinin in retaining freshness and green color of cabbages by applying N^6-benzyladenine prior to display in the market. It was also found that cabbages stored under root cellar conditions responded well to kinin treatment.

The marketability of superior storage types such as the Pennstate Ballhead, Danish Ballhead, and Jumbo varieties was extended by approximately one month under 40° F storage when treated with 30 - 40 ppm N^6-benzyladenine. This kinin appears to provide practical benefits in lengthening storage life and maintaining quality of cabbages stored or shipped under less than ideal conditions.

Experiments with radishes (bunched) indicated that a thermal-kinin interaction appears to exist and that kinin may affect a temperature-sensitive system such as an enzyme system. Since recent reports suggest that kinins appear to be associated with RNA metabolism in the control of senescence, RNase was postulated as a probable enzyme involved. In vitro studies indicated that one kinin, kinetin, appeared to have an inhibitory effect on the RNase degradation of s-RNA. These results strongly support the hypothesis that

kinins may inhibit the natural degradation of RNA by enzymes (possibly by the blockage of active sites or binding sites on the enzyme molecule), thereby maintaining the level of s-RNA for protein synthesis in the chloroplast and subsequently maintaining chlorophyll level.

The level of alkali-extractable RNA from Pennstate Ballhead cabbages treated with 50 ppm N^6-benzyladenine was found to be significantly higher than the RNA extracted from control cabbages. These results are in agreement with the published results of other workers on Xanthium and tobacco and support the hypothesis that kinins are implicated in the regulation of nucleic acid metabolism.

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